

CYTOSOLIC CHOLESTEROL ESTER HYDROLASE
IN ADRENAL CORTEX

by

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This thesis was composed by myself and the results therein are the product of my own work.

T O

M Y F A M I L Y

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ABSTRACT

Cholesterol ester hydrolase (CEH) in adrenocortical cytosol was known to be phosphorylated and activated, in response to ACTH in a cAMP-dependent protein kinase mediated process. The purification of CEH from bovine adrenocortical cytosol was attempted. The use of detergents to solubilise the enzyme from lipid-rich aggregates was investigated and sodium cholate was found to be effective. A purification procedure using cholate solubilised enzyme was developed. The detergent interfered in the operation of the ion-exchange columns, and pure enzyme was not obtained. Hydrophobic chromatography was studied but it was found to be unsuitable for the purification of CEH under the conditions investigated. An affinity chromatography technique was developed using columns of glass beads coated non-covalently with cholesterol oleate. Some positive results were obtained but the low capacity of the columns combined with the low concentration of the enzyme in the tissue cytosol prevented further study of the purified activity. A further purification procedure utilising a non-ionic detergent and gradient sievorptive chromatography resulted in a 150-fold purification of CEH from bovine adrenocortical cytosol with a recovery of about 25%. The specific activity of the partially purified enzyme (CEH_{Q2} preparation) was approximately 60 nmol oleic acid produced.min⁻¹.mg protein⁻¹. Labelling studies using the CEH_{Q2} preparation, $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP and $\left[1,3\text{-}^3\text{H}\right]$ DFP suggested that the enzyme activity was associated with a protein with Mr approximately 84 000.

A phosphoprotein phosphatase with Mr 35 000 was purified from bovine adrenocortical cytosol to a state approaching homogeneity. The purified phosphatase was active when measured towards ³²P-phosphoprotein and p-nitrophenyl phosphate. The role of this phosphoprotein phosphatase in the modulation of cytosolic CEH activity was investigated, but the enzyme did not deactivate partially-purified CEH from bovine adrenocortical cytosol.

Cytosolic CEH in rat adrenal was found to exhibit a diurnal variation in activity. The enzyme activity was significantly higher during the dark phase. Serum corticosterone concentration reflected this variation in CEH activity. In vivo experiments including acute ACTH administration and dexamethasone suppression of pituitary ACTH secretion suggested that ACTH was involved in the production and maintenance of the diurnal rhythm of rat adrenal cytosolic CEH activity.

The protein components of bovine adrenocortical and rat adrenal lipid droplets were investigated. The lipid droplets from both species contained a major protein subunit with Mr 40 000 and several other minor proteins. The protein profile was similar in both species. The Mr 40 000 apoprotein was able to be phosphorylated in rat lipid droplets but not in bovine lipid droplets. The delipidated apoprotein was phosphorylated in both cases. Bovine adrenocortical lipid droplets contained about 4% by weight protein, but CEH was found to be only a minor component of the protein fraction.

ABBREVIATIONS

The following abbreviations were used throughout the text:

ACTH	- Adrenocorticotrophic hormone, Corticotropin
Cholesterol	- 5-Cholesten-3 β -ol
CEH	- Cholesterol ester hydrolase
DFP	- Di-isopropyl phosphorofluoridate
DTT	- Dithiothreitol
h	- Hour(s)
(h)HDL	- (human) High density lipoprotein
HSL	- Hormone-sensitive lipase
LDL	- Low density lipoprotein
LH	- Luteinising hormone, lutropin
min	- minute(s)
Mr	- Molecular weight (relative molecular mass)
MSH	- Melanocyte stimulating hormone
NCS	- Nuclear Chicago Solubiliser - Tissue solubiliser
PAGE	- Polyacrylamide gel electrophoresis
s	- Second(s)
S.D.	- Standard deviation
SDS	- Sodium dodecyl sulphate
S.E.M.	- Standard error of the mean
TCA	- Trichloroacetic acid
TES	- N-tris (hydroxymethyl) methyl-2 aminoethane sulphonic acid
TEMED	- N,N,N',N'-tetramethylethylenediamine
VLDL	- Very low density lipoprotein
V _o	- Void volume

ENZYMES

- Adenylate cyclase or ATP pyrophosphate-lyase (cyclising) (EC 4.6.1.1)
- Alkaline phosphatase or orthophosphoric-monoester phosphohydrolase (alkaline optimum) (EC 3.1.3.1)
- Cholesterol acyltransferase or acyl-CoA:cholesterol O-acyltransferase (ACAT) (EC 2.3.1.26)
- Cholesterol ester hydrolase (CEH), cholesterol esterase or sterol-ester acylhydrolase (EC 3.1.1.13)
- Cholesterol 7 α -monooxygenase, cholesterol 7 α -hydroxylase or cholesterol, NADPH:oxygen oxidoreductase (7 α -hydroxylating) (EC 1.14.13.17)
- Cholesterol 20-hydroxylase or cytochrome P-450_{scc} (EC 1.14.1.9)
- Glyceraldehyde-phosphate dehydrogenase or D-glyceraldehyde-3-phosphate: NAD⁺ oxidoreductase (phosphorylating) (EC 1.2.1.12)
- Hydroxymethylglutaryl-CoA reductase (NADPH), HMGCoA-reductase or mevalonate:NADP⁺ oxidoreductase (CoA-acylating) (EC 1.1.1.34)
- Lecithin-cholesterol acyltransferase (LCAT) or lecithin:cholesterol acyl transferase (EC 2.3.1.43)
- Phosphoglycerate kinase or ATP:3-phospho-D-glycerate 1-phosphotransferase (EC 2.7.2.3)
- Phosphoprotein phosphatase or phosphoprotein phosphohydrolase (EC 3.1.3.16)
- Protein kinase or ATP:protein phosphotransferase (EC 2.7.1.37)
- Triacylglycerol lipase, hormone-sensitive lipase (HSL) or triacylglycerol acylhydrolase (EC 3.1.1.3)

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SECTION 1

INTRODUCTION

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1.1 GENERAL INTRODUCTION

Cholesterol has been shown to play an important role as the sole precursor for the steroid hormones produced in the adrenal cortex, gonads and placenta (Sandor, Fazekas and Robinson (1976), Dufau and Catt (1978), Simpson and Mason (1979)). The adrenal steroids fall into two main groups. The mineralocorticoids, such as aldosterone, have a primary effect on the excretion of Na^+ and K^+ and so regulate electrolyte and water balance. The glucocorticoids, such as cortisol and corticosterone, exert major effects on carbohydrate, fat and protein metabolism and play a vital but poorly-understood role in the response to stress. After adrenalectomy an animal is in a very unstable metabolic condition and soon dies in response to the slightest stress. There are also several diseases resulting from adrenal hypofunction (Addison's disease) and hyperfunction (Cushing's disease or Cushing's syndrome) (see James (1975), Baxter and Rousseau (1979)).

The first and rate limiting step in the process of steroidogenesis is the cholesterol side chain cleavage reaction which occurs in the inner cristae of the mitochondria (Stone and Hechter (1954)). The reaction is catalysed by a cytochrome P-450-dependent mixed function oxygenase (cytochrome P-450_{scc}) and involves sequential hydroxylations in the sterol side chain at C_{22} and C_{20} followed by cleavage of the cholesterol side chain between C_{20} and C_{22} to yield the key intermediate pregnenolone and isocaproic aldehyde (McKerns (1968), Burstein, Kimball and Gut (1970), Gower (1975)). In the adrenal cortex the pregnenolone is further metabolised in the endoplasmic reticulum by 17α -hydroxylation and or 21 -hydroxylation and by oxidation of the 3β -hydroxysteroid Δ -5 structure to the 3-keto Δ -4 structure to form deoxycortisol and deoxycorticosterone. The differential production of these steroids is partly dependent upon the species (Sandor, Fazekas

and Robinson (1976)). Deoxycortisol and deoxycorticosterone may then be transported back into the mitochondria for 11β -hydroxylation to form cortisol and corticosterone and in the zona glomerulosa, 18-hydroxylation may also occur to form aldosterone.

The process of steroidogenesis in the adrenal cortex is stimulated by ACTH (reviewed Schulster (1974), Halkerston (1975), Haynes (1975), Gill (1979), Tait, Tait and Bell (1980)). The mechanisms involved in the ACTH-induced stimulation of steroidogenesis have been vigorously investigated during the past twenty-five years. It was found that incubation of bovine adrenocortical slices with ACTH resulted in a marked increase in cAMP concentration in this tissue (Haynes (1958)). Subsequently, it was shown that the addition of cAMP in vitro to rat adrenal slices resulted in an increased corticoid production that was similar to the effect of ACTH on this tissue (Haynes, Koritz and Peron (1959)). These initial observations were confirmed by several other studies (see Halkerston (1975)) and the role of cAMP as the intracellular secondary messenger for the ACTH stimulus was established. Investigation of the mechanism of action of cAMP led to the discovery of a specific cAMP-binding protein in the cytosol and microsomes of adrenal cortex (Gill and Garren (1969)). Following the discovery of rabbit skeletal muscle cAMP-dependent protein kinase (Walsh, Perkins and Krebs (1968)), a protein kinase from bovine adrenal cortex cytosol was identified and it was shown that its activity was enhanced by cAMP (Gill and Garren (1970)). The relationship between the cAMP-activatable protein kinase and the previously discovered cAMP-binding protein was subsequently elucidated (Gill and Garren (1970), Gill and Garren (1971)) and the mechanisms by which the nucleotide activates the cAMP-dependent protein kinase are now well understood (see Flockhart and Corbin (1982)).

The free catalytic-subunit of cAMP-dependent protein kinase catalyses the transfer of the terminal phosphate group of ATP to various protein acceptors and in this way can alter the activity or function of the protein. The diverse manifestations of cAMP in different tissues results from the phosphorylation of tissue-specific proteins by the activated protein kinase (see Lincoln and Corbin (1978)). The specific protein phosphorylated in adrenal cortex was not known. It is now accepted that certain criteria should be satisfied to establish that a given enzyme undergoes physiologically significant phosphorylation-dephosphorylation. The criteria, as formulated by Krebs (1972) and later modified (Krebs and Beavo (1979)) can be summarised as follows. The enzyme in question should be phosphorylated in vitro at a significant rate in a protein kinase mediated reaction; the enzyme should be dephosphorylated by a phosphoprotein phosphatase; the enzyme should also show significant changes in its functional properties and these should correlate with the degree of phosphorylation. Furthermore, the most difficult criterion which has been met by only very few enzymes, so far, demands that the phosphorylation-dephosphorylation of the enzyme occurs in vivo and correlates with functional changes.

Cholesterol is stored in the adrenal cortex as large cholesterol ester rich lipid droplets in the cytoplasm (Garren, Gill et al. (1971)). It was known that administration of ACTH to animals resulted in a reduction of the cholesterol content of the adrenal cortex (Sayers, Sayers et al. (1944)) and this was due, almost entirely, to a decrease in the esterified cholesterol (Davis and Garren (1966)). The mobilization of cholesterol esters in response to ACTH was not affected by prior injection of cycloheximide, an inhibitor of protein synthesis in the cytosol which blocks ACTH stimulation of adrenal steroidogenesis. It was discovered that administration of ACTH to rats, 15 min before the

adrenals were removed, led to an activation of cholesterol ester hydrolase (CEH) in the adrenal homogenate (Shima, Mitsunaga and Nakao (1972)). Approximately 70% of the neutral CEH activity was associated with the cytosol, and thus was present in the same subcellular compartment as the lipid droplets (Trzeciak and Boyd (1973)). Ether anaesthesia stress, which is known to increase the ACTH concentration in the blood (Matsuyuna, Ruhmann-Wennhold and Nelson (1971)), has been shown to activate cytosolic CEH and produce cholesterol ester depletion in rat adrenal lipid droplets (Trzeciak and Boyd (1973)). Administration of cycloheximide did not affect these observations, but there was an increase in free cholesterol in the lipid droplets (Boyd and Trzeciak (1973)). This was consistent with previous findings that cycloheximide did not affect the ACTH-induced increase in intracellular cAMP (Grahame-Smith, Butcher et al. (1967)). Incubation of rat adrenal cytosol in vitro with cAMP and ATP resulted in the activation of CEH (Trzeciak and Boyd (1973)). The cAMP-dependent activation of adrenocortical cytosolic CEH has been confirmed by several other studies (Trzeciak and Boyd (1974), Naghshineh, Treadwell et al. (1974), Beckett and Boyd (1975), Wallat and Kunau (1976), Beckett and Boyd (1977), Pittman and Steinberg (1977), Naghshineh, Treadwell et al. (1978)). The activation was dependent upon the presence of cAMP-dependent protein kinase (Beckett and Boyd (1977), Naghshineh, Treadwell et al. (1978)) and inhibited by the heat-stable inhibitor of cAMP-dependent protein kinase (Beckett and Boyd (1977)). Incubation of impure preparations of the enzyme in vitro with cAMP and $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP led to the activation of the enzyme with concomitant phosphorylation of the protein fraction (Trzeciak and Boyd (1974), Beckett and Boyd (1977), Wallat and Kunau (1976), Naghshineh, Treadwell et al. (1978)). Furthermore, incubation of the ^{32}P -labelled enzyme fraction with Mg^{2+}

(Trzeciak and Boyd (1974), Wallat and Kunau (1976)) or exogenous alkaline phosphatase (Beckett and Boyd (1977)) led to a decrease in the enzyme activity with a concomitant loss of ^{32}P from the protein fraction. These studies led to the conclusion that ACTH stimulated steroidogenesis in the adrenal cortex via cAMP by the cAMP-dependent protein kinase-mediated phosphorylation and activation of cytosolic CEH. This results in the hydrolysis of cholesterol esters in the lipid droplets and an increase in the free cholesterol available to the rate limiting side chain cleavage reaction.

The situation with CEH in other steroidogenic tissues is less clearly defined. Beckett (1975) found that LH administration in vivo caused a depletion of esterified cholesterol in the lipid droplets from rat corpora lutea, associated with a two-fold stimulation of cytosolic CEH. Dibutyryl cAMP, when added to bovine corpus luteum cytosol, gave an activation of CEH (Bisgaier, Treadwell and Vahouny (1979)). However, it had also been shown that LH addition to bovine corpora lutea did not produce any evidence for the activation of CEH (Goldstein and Marsh (1973)). Furthermore LH administration in vivo to pregnant mare serum gonadotropin-primed immature female rats did not stimulate CEH, but incubation of the ovarian cytosol with cAMP in vitro did activate CEH (Gorban (1980)). Cholesterol ester depletion upon LH administration in vivo has been demonstrated in rat and rabbit (Behrman and Armstrong (1969), Flint and Armstrong (1978)), and cAMP accumulation and the stimulation of cAMP-dependent protein kinase have been observed in rat ovarian tissue in response to LH (Mason, Schaffer and Toomey (1973), Lamprecht, Zor et al. (1973)). However in ovarian tissues, the different cell types and the physiological state of the organ complicate the interpretation of some of the published work.

Although little work has been carried out, there is no evidence to suggest CEH in the Leydig cell cytoplasm is activated by LH or cAMP. It has been shown recently that cytosolic, neutral CEH activity in certain macrophages can be activated by cAMP in a cAMP-dependent protein kinase mediated reaction (Khoo, Mahoney and Steinberg (1981)). The physiological significance of this activity in macrophages is not clear, although it may be involved in atherosclerotic lesions derived from macrophages. Finally the enzyme has been shown to be not activatable in liver tissue (Gorban (1980)). It could be argued that liver does not require a rapid activation of CEH as a rapid increase in cholesterol ester hydrolysis is not required as it is in the adrenal cortex.

1.2 ADRENAL STEROIDOGENESIS

1.2.1 Role of lipoproteins

Cholesterol has been established as the sole precursor for steroidogenesis. It was known that the bulk of adrenal cholesterol in man was derived from plasma cholesterol (Borkowski, Delcroix and Levin (1972)) and that ACTH enhanced the uptake of [^3H] cholesterol from plasma into rat adrenal cortex (Dexter, Fishman and Ney (1970)). Goldstein and Brown (1974) showed that cultured human fibroblasts possess a specific uptake mechanism for cholesterol in plasma lipoproteins. These cells had a specific receptor for the apolipoprotein B found in LDL and after binding to the cell the LDL was internalised by endocytosis and degraded by lysosomal action (Brown and Goldstein (1976)). The so-called LDL receptor pathway has subsequently been found in mouse Y-1 adrenal tumour cells (Faust, Goldstein and Brown (1977)), 4-APP treated-rat adrenal cortex (Balasubramaniam, Goldstein et al. (1977)), cultured bovine adrenocortical cells and fresh bovine adrenal membranes (Kovanen, Faust et al. (1979)) and human fetal adrenal membranes (Brown, Kovanen and Goldstein (1979)).

It has been shown that ACTH increases the number of LDL receptors in cultured mouse and bovine adrenal cells and human fetal adrenal membranes in vitro (Faust, Goldstein and Brown (1977), Kovanen, Faust et al. (1979), Ohashi, Carr and Simpson (1981a)). The number of receptors are also increased some 5-fold in mice and rats in vivo when the animals are rendered lipoprotein deficient. The receptor has been partially purified from bovine adrenal cortex, which has one of the highest LDL receptor densities of all tissues, and antibodies have been prepared against it (Schneider, Goldstein and Brown (1980)). The LDL receptor can also bind apolipoprotein E and so lipoproteins with this apoprotein can also be taken up via this pathway (Mahley and Innerarity (1977)). However in rats the LDL concentration is low and 70-80% of plasma cholesterol is carried in HDL. It was found that rat adrenal cells can take up HDL, including human HDL which has no apolipoprotein E, by a receptor-mediated mechanism that was stimulated by ACTH (Gwynne and Hess (1980)). Human fetal adrenal membranes have also been shown to contain specific, saturable binding sites for HDL (Ohashi, Carr and Simpson (1981b)). Further kinetic studies on the uptake of lipoproteins have shown that rat adrenal can take up both LDL and HDL cholesterol, by separate mechanisms, and that HDL was the major source of adrenal cholesterol in this species (Anderson and Dietschy (1981)). The mechanism of HDL uptake is not known, but it is not via endocytosis and total degradation (Gwynne and Hess (1980)).

With the evidence obtained from these and several other studies a working model for cholesterol homeostasis in adrenal cortex has been formulated (see Brown, Kovanen and Goldstein (1979)). In this model, shown schematically in Fig. 1.1, adrenal cholesterol is considered to be in three pools. There is a fixed pool of free cholesterol in cell membranes, a metabolically active, rapidly turning over pool of free

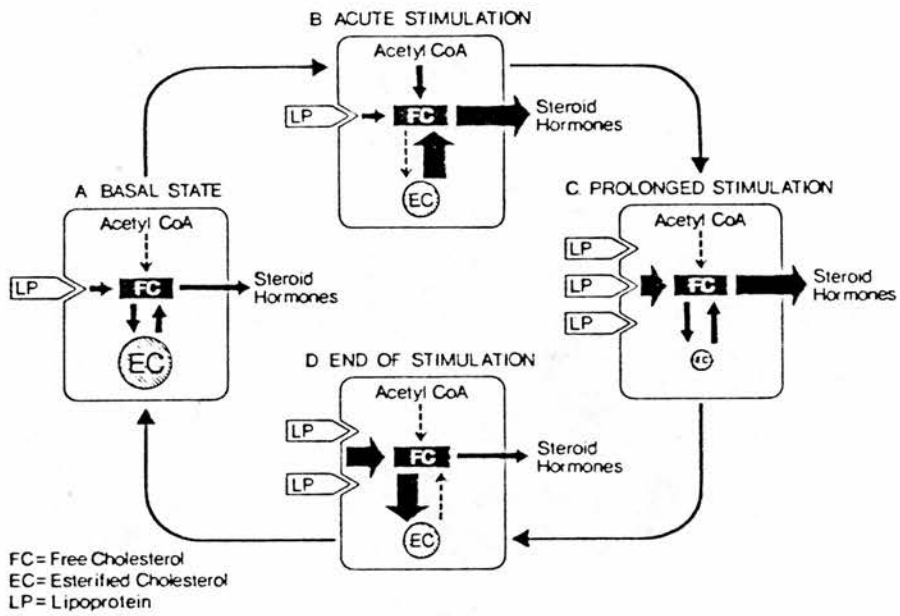


Fig. 1.1. Model for cholesterol homeostasis in the adrenal gland. The model shows sequential changes in cholesterol input and output during acute and prolonged stimulation of steroid secretion.

(Taken from Brown, Kovanen and Goldstein (1979)).

cholesterol (FC) and stored cholesterol, esterified to fatty acids in the lipid droplets (EC). Net output from FC is via steroidogenesis and secretion of steroids and net input to FC can come from de novo synthesis, plasma lipoprotein uptake or hydrolysis of cholesterol ester. Upon acute stimulation by ACTH, the rapid increase in cholesterol utilization for steroidogenesis is met by a large and rapid increase in cholesterol ester hydrolysis via activation of CEH. There may also be a slight stimulation of de novo synthesis via activation of HMGCoA-reductase (and HMGCoA synthase in the rat). If the stimulation is prolonged the EC stores will be depleted and the cholesterol will now be supplied by plasma lipoproteins via increased uptake due to induction of receptors. As the stimulus ceases there will be a transient phase where uptake from lipoproteins will exceed steroid hormone output, leading to stimulation of microsomal ACAT activity by membrane cholesterol, increased esterification and build up of EC stores. Normal turnover of receptors leads to a return to the basal state where a dynamic equilibrium probably exists with the low level of steroid hormone output balanced by input from lipoprotein uptake.

1.2.2 The role of ACTH and pituitary peptides

The stimulation of steroidogenesis by ACTH does not require the entrance of the ACTH polypeptide into the adrenal cell (Schimmer, Ueda and Sato (1968), Richardson and Schulster (1972)). These studies indicated the existence of a specific receptor or receptors on the external surface of the adrenal cell plasma membrane that bound the ACTH molecule. ACTH is a single polypeptide chain of 39 amino-acids, although only the N-terminal 24 are required for full steroidogenesis (Schwyzer (1977), Seelig and Sayers (1973)). Durand and Locatelli (1980) have shown that the number of ACTH receptors in rabbit adrenals decreases after hypophysectomy and receptor numbers are restored by

administration of ACTH. However, studies with ^{125}I -labelled ACTH indicated that there are 'spare receptors' as it was possible to get further ACTH binding to rat adrenocortical cells after maximal steroidogenesis was attained (McIlhinney and Schulster (1975)). Scatchard analysis in the same study also suggested two types of receptor, but a recent study using ^{125}I -labelled ACTH_{1-38} , that was biologically active with a high specific activity, found only one class of binding site (Buckley and Ramachandran (1981)).

The interaction of ACTH with its receptor may involve the activation of membrane phospholipase and alteration of the membrane phospholipids, or changes in the sequestration of membrane calcium (see Rubin and Laychock (1978)). The hormone-receptor complex on the external surface of the cell membrane interacts with and activates the catalytic subunit of adenylate cyclase, located on the inner surface of the cell membrane, leading to the production of cAMP from ATP (see Rodbell (1978)). The activation of the catalytic subunit of adenylate cyclase by the hormone-receptor complex is mediated by the guanine nucleotide-binding regulatory protein. Bound GTP is required for full activity of adenylate cyclase, and the subsequent hydrolysis of GTP to GDP is required to 'switch-off' the activity (Rodbell (1978), Ross and Gilman (1980)). Cholera toxin stimulates cAMP accumulation and steroidogenesis in isolated rat adrenocortical cells (Palfreyman and Schulster (1975)) by ADP-ribosylating a 41 000-45 000 Mr polypeptide in the GTP binding protein, preventing hydrolysis of GTP and so activating the adenylate cyclase (Ross and Gilman (1980)). Recently, a 68 000 Mr protein has been implicated in the coupling of the ACTH hormone receptor to the adenylate cyclase complex (Watt and Schimmer (1981)).

The release of ACTH from the pituitary is stimulated by corticotropin-releasing factor (CRF) (Turkelson, Arimura et al. (1981), possibly via a cAMP-dependent process (Labrie, Vielleux et al. (1982)).

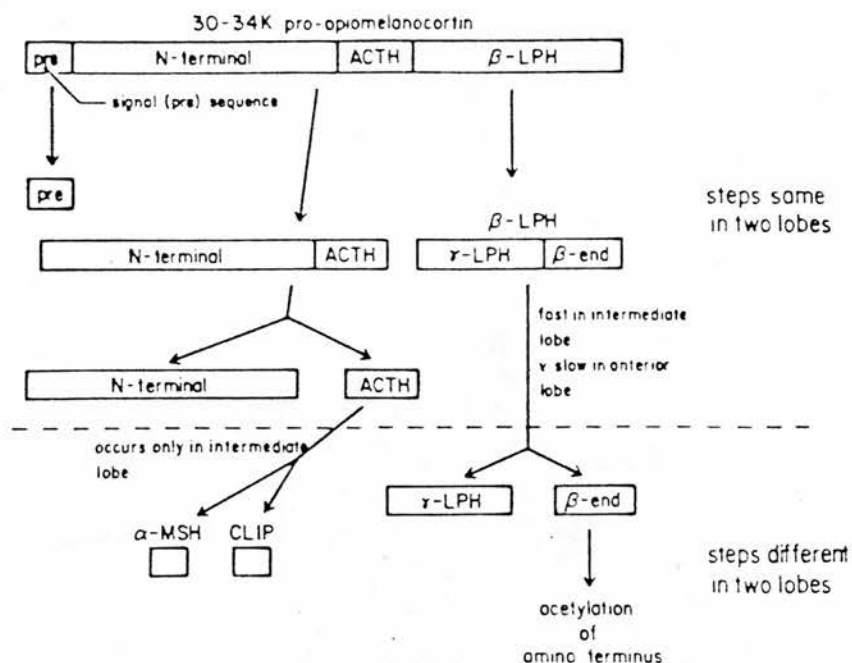
It now appears that CRF has been identified as a 41 amino-acid peptide (Vale, Spiess et al. (1981)), and has been purified, sequenced and synthesised by solid-phase techniques.

In the anterior pituitary, ACTH is initially synthesised as part of a larger molecule with Mr 30 000-34 000 called pro-opiomelanocortin (POMC) (see Herbert (1981)). This molecule is a polypeptide containing the sequences of MSH, ACTH and β -endorphin, and is first cleaved into an ACTH containing fragment and β -lipotropin (β -LPH). In the anterior lobe of the pituitary the ACTH sequence is cleaved from the former fragment but in the intermediate lobe the ACTH, so produced, can be cleaved further to form α -MSH. Similarly in the intermediate lobe, β -lipotropin is cleaved to form γ -lipotropin (γ -LPH) and β -endorphin (see Fig. 1.2a).

Pederson and Brownie (1979) found that in stressed rats, CEH activity in adrenal cytosol was increased along with increased steroidogenesis, but in hypophysectomised rats, injections of purified ACTH₁₋₃₉ or ACTH₁₋₂₄ gave increased steroidogenesis without activation of CEH. This was in contrast to previous studies where hypophysectomy in rats reduced CEH activity in the adrenal and ACTH administration restored the enzyme activity to the control level (Behrman and Greep (1972)) or above (Trzeciak, Mason and Boyd (1979)). Further work investigating various peptides derived from the 31 000 precursor showed that the N-terminal end prior to the ACTH sequence (16K fragment), in similar amounts to ACTH, had a slight effect in potentiating ACTH₁₋₂₄ action, and that trypsin treatment of the 16K fragment increased this effect (Pederson and Brownie (1980)). Administration of the trypsin digest alone to hypophysectomised rats resulted in an apparent increase in adrenal CEH activity but no activation of side chain cleavage, whereas when added with ACTH₁₋₃₉

a

COMPARISON OF PROCESSING IN
ANTERIOR AND INTERMEDIATE LOBES



b

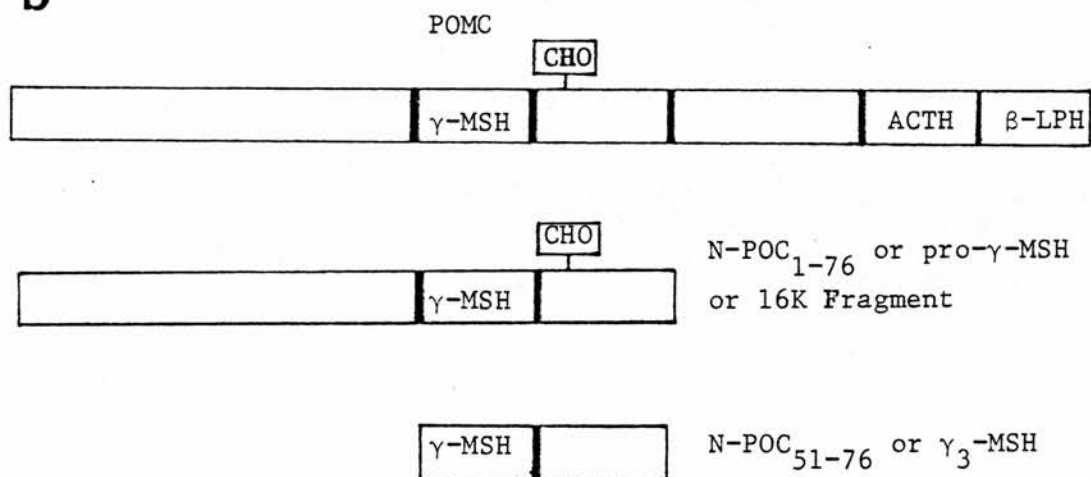


Fig. 1.2. a) The processing of the Mr 30 000-34 000 pro-opiomelanocortin (POMC) molecule in the pituitary gland.

(Taken from Herbert (1981)).

b) Relationship of the POMC molecule to some of the peptides derived from it. The 16K fragment has an apparent Mr approximately 16 000 on SDS/PAGE. Amino acid analysis has shown this molecule is identical to N-POC₁₋₇₆ with Mr approximately 12 000.

(Taken from Estivariz, Iturriza et al. (1982)).

there was an increase in both adrenal CEH activity and serum corticosterone. It was subsequently discovered that a synthetic peptide of 27 amino-acids (a portion of the 16K fragment, also termed γ_3 -MSH or N-POC₅₁₋₇₆, see Fig. 1.2b) containing the γ -MSH sequence plus a carboxyl terminal extension was responsible for the stimulation of CEH activity (Pederson, Brownie and Ling (1980)). These workers postulated that in stress, ACTH₁₋₃₉ and another peptide, derived from the POMC molecule, were necessary for the full stimulation of steroidogenesis in the adrenal cortex.

Another study has found that circulating human pituitary γ -MSH precursor (pro- γ -MSH or N-POC₁₋₇₆, see Fig. 1.2b) at nanomolar concentrations, potentiated and enhanced ACTH action in perfused rat and human adrenal cells (Al-Dujaili, Hope et al. (1981)), but this effect was blocked by transcription inhibitors such as actinomycin D (Al-Dujaili, Williams et al. (1982)). It has also been found that α -MSH and β -MSH stimulate corticoid production in rat capsular cells (Vinson, Whitehouse et al. (1980), Matsuoka, Mulrow et al. (1981)) and decapsular cells (Li, Ng and Cheng (1982)), although at concentrations about 1000 x greater than ACTH. The physiological significance and the possible role of these peptides in stimulating adrenal steroidogenesis are not known.

1.2.3 The role of cAMP

The role of cAMP as the secondary, intracellular mediator of the action of ACTH in the adrenal cortex was established by Grahame-Smith, Butcher et al. (1967). However further studies produced some conflicting evidence that may be summarised briefly as follows: In isolated adrenal cells or perfusion systems, the correlation was poor between the rise in cAMP and increasing steroidogenesis at low or high ACTH concentrations (Mackie, Richardson and Schulster (1972), Charchman, Jaanus and Rubin (1971), Hudson and McMartin (1975)). Analogues of ACTH

such as NPS-ACTH (o-nitrophenylsulphonyl derivative) could give maximal steroidogenesis with little effect on cAMP (Moyle, Kong and Ramachandran (1973)). Low levels of ACTH that did not appear to activate protein kinase could give increased steroidogenesis (Richardson and Schulster (1973)).

These observations suggested that the cAMP secondary messenger concept in its simplest form may not be a full explanation of acute stimulation of steroidogenesis by ACTH in the adrenal cortex (Ramachandran and Moyle (1977)). However there are several possible reasons for the discrepancies observed in some experiments.

There are deficiencies in certain cAMP assays, and furthermore, stimulated adrenal cells generally produce more cAMP than is needed for complete protein kinase activation and maximal steroidogenesis (Podesta, Milani et al. (1979a)). It is also possible that there may be two populations of ACTH receptors with differing affinities, as suggested by Scatchard analysis (McIlhinney and Schulster (1975)). Furthermore, it has been suggested that there may be spare receptors so that there could be more ACTH receptors than are required for maximal steroidogenesis (McIlhinney and Schulster (1975)). It has been shown in isolated adrenal cells that bound cAMP correlates well with steroidogenesis at low concentrations of ACTH (Podesta, Milani et al. (1979a)). Recently a mathematical model was produced that suggested there may be an obligatory separation of hormone binding and biological response curves in systems dependent upon secondary mediators (Strickland and Loeb (1981)).

Some investigators have found that ACTH stimulated cGMP production in isolated rat adrenal cells with no increase in cAMP (Sharma, Ahmed and Shanker (1976), Perchellet, Shanker and Sharma (1978)), but this was contrary to all previous evidence. Subsequent studies have again

shown that ACTH caused a rise in intracellular cAMP while cGMP levels remained virtually unchanged (Laychock and Hardman (1978), Saez, Evain and Gallet (1978), Podesta, Milani et al. (1979a)).

Recently, further work using ACTH analogues that were either agonists or antagonists to ACTH has suggested that, of the two possible classes of ACTH receptor (McIlhinney and Schulster (1975)), only one operates via the cAMP-dependent mechanism while the other operates through some other unknown mechanism (Bristow, Gleed et al. (1980)). However Rae, Gutmann et al. (1979) have shown the obligatory need for cAMP-dependent protein kinase in ACTH-stimulated steroidogenesis in adrenal cells. Therefore, although most of the evidence is in agreement with cAMP as the major intracellular mediator of ACTH action in adrenal cells, there may be other factors involved at the secondary messenger level in response to ACTH, such as calcium (Rubin, Jaanus and Charchman (1972), Mahaffee and Ontjes (1980), Podesta, Milani et al. (1980), Garcia, Laychock and Rubin (1982)) or cGMP (Guillemant and Guillemant (1981)).

1.2.4 The role of cAMP-dependent protein kinase and other protein kinase activities

Since the discovery by Gill and Garren (1970) of a cAMP-dependent protein kinase in adrenal cortex, and subsequently, the importance of CEH as a major substrate for the kinase in this tissue (Trzeciak and Boyd (1974)), several other protein kinase activities have been found in adrenal cortex. Both type I and type II cAMP-dependent protein kinase activities have been isolated from and characterised in bovine adrenal cortex, but the possible differential roles of each activity in ACTH-stimulated steroidogenesis are not known (Ebert and Finn (1981)). The heat-stable inhibitor protein (HSIP) of cAMP-dependent protein kinase was also found in adrenal cortex (Cochet, Job and Chambaz (1977b)).

A cGMP-dependent protein kinase has also been purified from bovine adrenal cortex (Ahrens, Paul et al. (1982)). This enzyme had the same structure as other cGMP-dependent protein kinases (see Flockhart and Corbin (1982)) and was activated by cGMP, its analogues and possibly also by calmodulin. Several cyclic nucleotide-independent protein kinase activities have been isolated from bovine adrenal cortex since the initial discovery of a cAMP-independent protein kinase that preferentially phosphorylated casein (Cochet, Job and Chambaz (1977a&b)). These investigators have now isolated four cyclic nucleotide-independent protein kinase activities that phosphorylated casein, including one that utilised ATP as a phosphate donor (CKA) and three that could use either ATP or GTP (CKG) (Cochet, Job et al. (1980)). A specific heat-stable inhibitor protein (CKG I) of the CKG type was also found (Job, Cochet et al. (1979)) and the effect of the inhibitor could be reversed by polyamines (Job, Pirollet et al. (1979)). Further studies have shown that naturally occurring glycosaminoglycans can inhibit the CKG type (Pirollet, Feige et al. (1980), Feige, Pirollet et al. (1980)).

A protein kinase (PK380), whose activity was independent of cyclic nucleotides, Ca^{2+} or calmodulin, and that specifically phosphorylated an endogenous 120 000 Mr protein and eukaryotic initiation factor 2α (eIF- 2α), was found in bovine adrenal cortex (Kuroda and Sharma (1980)). The PK380-catalysed phosphorylation of eIF- 2α was found to be inhibited by polyamines such as spermine, spermidine and putrescine (Kuroda, Merrick and Sharma (1982)). A further study showed that incubation of intact adrenocortical cells with $[\gamma\text{-}^{32}\text{P}]$ ATP resulted in the phosphorylation of two endogenous proteins with Mr 39 000 and 76 000, and after addition of ACTH, a further protein with Mr 87 000 was phosphorylated (McPherson and

Ramachandran (1980)). This protein kinase activity was found to be cyclic nucleotide-independent and was associated with the outer surface of the plasma membrane. A cAMP-dependent protein kinase, that is an integral protein in the plasma membrane of bovine adrenocortical cells has also been described (Reitherman, Chen et al. (1981)).

Takai, Kishimoto et al. (1977) described a protein kinase activity in rat brain tissue that was independent of cyclic nucleotides, phosphorylated histone and protamine, and was activated by limited proteolysis with Ca^{2+} -dependent protease. The activated kinase (protein kinase C) could phosphorylate phosphorylase kinase and glycogen synthase. Subsequently, it was found that the protein kinase could be activated without proteolysis by Ca^{2+} and phospholipids (Takai, Kishimoto et al. (1979a)). Unsaturated diacylglycerol sharply decreased the Ca^{2+} and phospholipid concentrations required for full activation (Takai, Kishimoto et al. (1979b)). Phosphatidyl serine was established as the sole phospholipid effective for the activation, although other phospholipids could modulate this effect (Kaibuchi, Takai and Nishizuka (1981)). This Ca^{2+} -activated, phospholipid-dependent protein kinase has been demonstrated in heart (Limas (1980)), platelets (Takai, Kaibuchi et al. (1981)), pancreas, liver, vas deferens and adrenals (Wrenn, Katoh and Kuo (1981)). It was shown that in adrenal 30 000 x g supernatant, phosphatidyl serine/ Ca^{2+} stimulated the phosphorylation of proteins with Mr 49 000, 18 000 and 13 000, whereas there was no calmodulin/ Ca^{2+} -dependent phosphorylation (Wrenn, Katoh and Kuo (1981)). However, there was Ca^{2+} /calmodulin-dependent protein kinase activity found in adrenal membranes in a previous study (Schulman and Greengard (1978)).

The role of the Ca^{2+} -activated, phospholipid-dependent protein kinase in an integrated cellular control system in platelets has been

discussed (Michell (1981)), but the role of this kinase in basal or ACTH-stimulated steroidogenesis in the adrenal cortex is not known at present. Similarly, the roles, if any, of cyclic nucleotide-independent protein kinases in adrenal steroidogenesis are unknown. However, possible roles for cGMP-dependent protein kinase (Ahrens, Paul et al. (1982)) and membrane bound protein kinase (Bristow, Salmon and Schulster (1981)) have been postulated.

1.2.5 The role of protein synthesis and polyphosphoinositides

It had been known for some time that inhibitors of cytosolic protein synthesis such as puromycin or cycloheximide, inhibit ACTH-stimulated steroidogenesis (Ferguson (1963), Garren, Ney and Davis (1965)). This led to the belief that some rapidly turning over, labile protein was involved in ACTH-stimulated steroidogenesis, and that ACTH, in some way stimulated its synthesis. The role of this protein and the mechanism by which ACTH stimulates its formation are still unclear. A major site of stimulation by ACTH in adrenal steroidogenesis is the mitochondrial, side chain cleavage reaction. This is probably due to increased association of substrate cholesterol with cytochrome P-450_{scc} rather than an intrinsic effect on the activity of the components of the enzyme system (see Tait, Tait and Bell (1980)). It was thought that the labile protein may be a "cholesterol translocase" involved in the transport of free cholesterol to the inner mitochondrial membrane (Simpson and Mason (1979)) but the overall evidence now suggests that the labile protein may be involved in the binding of cholesterol to cytochrome P-450_{scc} (Koritz and Hall (1964)).

It was known that cAMP-dependent protein kinase phosphorylated adrenocortical ribosomal proteins in vitro (Walton and Gill (1973)). Furthermore, it has been shown that an Mr 150 000 polypeptide in adrenocortical polyadenylated messenger ribonucleoprotein complex was

phosphorylated by endogenous cAMP-dependent protein kinase (Moore and Sharma (1980)). In both cases the phosphorylation of these proteins may lead to some translational control, resulting in the increased synthesis of the labile protein in response to the ACTH stimulus. However, evidence from experiments with perfused rat adrenocortical cells prompted the authors to suggest that the labile protein may exist as a stable, inactive precursor, with cAMP converting it, possibly via phosphorylation, into an active but unstable form (Lowry and McMartin (1974)). Some recent work on LH- and cAMP-stimulated steroidogenesis in isolated rat Leydig cells has provided evidence that this may be the mechanism operating in gonadotropin-stimulated steroidogenesis (Cooke, Lindh and Van Der Molen (1979)).

There is no evidence for the identity, in molecular terms, of the labile protein. In general, in vivo labelling studies with ACTH and labelled amino-acids have only given information about the long-term trophic effect of the hormone. Incubation of adrenal slices with labelled leucine and ACTH showed a general trophic effect on most proteins, with marked stimulation of a cytosolic protein with Mr 30 000 (Dazord, Gallet and Saez (1978)) and a mitochondrial protein with Mr 134 000 (Dazord, Gallet de Santerre and Saez (1981)). A similar experiment with mouse Y-1 adrenocortical cell cultures showed amino-acid incorporation into eight mitochondrial proteins, six of which were translated in the cytoplasm including possibly, adrenodoxin and its reductase (Ray, Horst and Kowal (1980)). Using cultured bovine adrenocortical cells it has also been shown that long-term ACTH stimulation induced the synthesis of mitochondrial cytochrome P-450_{scc} first as a 54 500Mr precursor, possibly by an increase in the transcription of messenger RNA (DuBois, Simpson et al. (1981)).

Recently, however, a series of experiments have suggested that the cycloheximide-sensitive factor may be involved in phospholipid metabolism. It was known that polyphosphoinositides were implicated in the regulation of membrane permeability and synaptic transmission in neurons, and possibly many other membrane events (Michell (1975)). Furthermore ACTH was known to affect polyphosphoinositide metabolism in rat brain membranes (Jolles, Wirtz et al. (1979)). It was also reported that ACTH increased adrenal polyphosphoinositides and it was suggested that they could stimulate cholesterol side-chain cleavage via the polyphosphorylated head group in a Ca^{2+} -dependent process (Farese, Sabir and Vandor (1979), Farese and Sabir (1979)). To be an intermediary in the ACTH stimulatory pathway, the polyphosphoinositides must fulfil certain criteria such as rapid synthesis and degradation preceding rapid changes in steroidogenesis in response to ACTH; a sustained increase during prolonged ACTH; graded increases paralleling graded steroidogenesis in response to doses of ACTH; an increase in response to stress; an increase in response to cAMP; dependence upon Ca^{2+} ; dependence on protein synthesis. A subsequent study claimed that polyphosphoinositides fulfilled these criteria and it was postulated that the ACTH-induced rise in cAMP caused an increase in polyphosphoinositides (along with activation of protein kinase) and these stimulated the cholesterol side-chain cleavage reaction (Farese, Sabir et al. (1980)).

The cAMP-induced rise in polyphosphoinositides was inhibited by cycloheximide. Further labelling studies (Farese, Sabir and Larson (1980a), Farese, Sabir and Larson (1981a)) and kinetic studies (Farese, Sabir and Larson (1980b), Farese, Sabir and Larson (1981b)) showed that ACTH stimulated a rapid rise in phosphatidic acid and phosphatidyl inositol that required Ca^{2+} and was blocked by cycloheximide.

However the conversion of phosphatidic acid to polyphosphoinositide was rapid and not inhibited by cycloheximide, and so the cycloheximide-sensitive factor was postulated to be a rapidly turning over protein involved in the de novo synthesis of phosphatidic acid. Similar work has been performed with bovine luteal cells and LH, producing similar results (Davis, Farese and Marsh (1981)). However, cAMP has not always mimicked the effects of the hormones in these experiments (Farese, Sabir and Larson (1980a), Davis, Farese and Marsh (1981)). Furthermore, whereas the ACTH stimulation of phosphatidic acid concentration appeared to be due to an increase in the diacylglycerol precursor, the cycloheximide inhibition appeared to be due to decreased diglyceride kinase (Farese, Sabir and Larson (1981c)).

Therefore the identity of the cycloheximide-sensitive factor, or labile protein, is still unclear, as are the main mechanisms involved in ACTH or cAMP induction of the protein. However, the possibility that ACTH may stimulate the formation of diacylglycerols may lead to the discovery of a role for the Ca^{2+} -sensitive, phospholipid-dependent protein kinase discussed in Section 1.2.4.

1.2.6 The role of protein phosphorylation

The classical pathway for cAMP-mediated regulation is via protein and enzyme phosphorylation through the action of cAMP-dependent protein kinase (see Krebs and Beavo (1979), Cohen, (1980), Flockhart and Corbin (1982)). Hardie (1981) reviewed the enzymes involved in lipid biosynthesis that can be phosphorylated, although not all were regulated by cAMP. In the adrenal cortex it was discovered that CEH was phosphorylated and activated by cAMP-dependent protein kinase in response to ACTH or cAMP (Trzeciak and Boyd (1973)). The cAMP-dependent phosphorylations of ribosomal protein (Walton and Gill (1973)) and a protein in polyadenylated messenger ribonucleoprotein complex

(Moore and Sharma (1980)) have also been demonstrated and the possible role of these phosphorylations in ACTH-stimulated steroidogenesis was discussed in Section 1.2.5. However, many other proteins are known to be phosphorylated in adrenal cortex in response to ACTH or cAMP.

Podesta, Milani et al. (1979b) have shown that incubation of intact, isolated adrenocortical cells with $^{32}\text{P}_i$ and ACTH or cAMP resulted in increased phosphorylation of a cytosolic protein with Mr 150 000. Receptor bound cAMP, corticosterone production and appearance of the phosphorylated protein all increased in parallel. Koroscil and Gallant (1980) incubated rat adrenal quarters with $^{32}\text{P}_i$ and ACTH, and found consistent and distinctive phosphorylation and dephosphorylation of specific proteins in cytosolic, microsomal and mitochondrial fractions. The changes in phosphorylation preceded the increase in corticosterone and the degree of phosphorylation was dose-dependent for ACTH and correlated well with corticosterone production. Furthermore, cAMP was found to give an identical effect to ACTH, whereas cGMP gave a pattern similar to the control. A comparative study of the ACTH effect on phosphorylation-dephosphorylation in vivo and in vitro was also performed where results obtained by injecting $^{32}\text{P}_i$ and ACTH into the intact animal were compared with results obtained from experiments with rat adrenal quarters or isolated cells (Koroscil and Gallant (1981)). Although fewer proteins were labelled in vivo the patterns obtained were very similar to the in vitro experiments. The phosphorylation-dephosphorylation effects in vitro were not affected by protein synthesis inhibitors which completely inhibited corticosterone production. Incubation of bovine adrenocortical plasma membrane fractions with $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP and ACTH or cAMP resulted in the enhanced phosphorylation of several proteins within 10 s (Bristow, Schulster and Rodnight (1981)).

The identities of all these proteins phosphorylated or dephosphorylated in response to ACTH and cAMP in adrenal cortex are not known and so the steroidogenically important phosphorylations have not been identified in these studies. Although it was known from several studies that CEH was modulated by phosphorylation in response to cAMP, the lack of a pure preparation had prevented the identification of the enzyme protein (Trzeciak and Boyd (1974), Wallat and Kunau (1976), Beckett and Boyd (1977)).

1.2.7 The role of cholesterol ester hydrolase (CEH)

Haynes (1958) showed that incubation of bovine adrenocortical slices with ACTH resulted in increased cAMP, and that the addition of cAMP in vitro to rat adrenal slices stimulated corticoid production similar to the effect of ACTH (Haynes, Koritz and Peron (1959)). A cAMP-dependent protein kinase was discovered in adrenocortical cytosol by Gill and Garren (1970), and this enzyme is now established as an obligatory component for full ACTH-stimulated steroidogenesis (Rae, Gutmann et al. (1979)). Cholesterol is stored in the adrenal in cytoplasmic lipid droplets, mainly as the ester of long-chain fatty acids (Moses, Davis et al. (1969), Beckett and Boyd (1975)). It was known that ACTH caused a reduction in the cholesterol content of the adrenal gland (Sayers, Sayers et al. (1944)) which was subsequently shown to be due almost entirely to a decrease in esterified cholesterol (Davis and Garren (1966)). It was then shown that rat adrenal CEH activity could be significantly enhanced by the prior injection of ACTH (Shima, Mitsunaga and Nakao (1972)). All these experimental observations were linked together when Trzeciak and Boyd (1973) showed that ether anaesthesia stress, which increases blood ACTH, stimulated the activity of CEH in rat adrenal cytosol and also the depletion of cholesterol esters in the lipid droplet fraction.

Nearly 70% of the CEH was found in the cytosol and incubation of the cytosol in vitro with cAMP and ATP led to an activation of the enzyme.

The cAMP-dependent activation of cytosolic CEH in adrenal cortex has been confirmed by several other studies (Trzeciak and Boyd (1979), Naghshineh, Treadwell et al. (1974), Wallat and Kunau (1976), Beckett and Boyd (1977)) and was shown to be dependent upon cAMP-dependent protein kinase (Trzeciak and Boyd (1974), Beckett and Boyd (1977), Naghshineh, Treadwell et al. (1978)). Incubation of partially purified CEH with $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP and cAMP resulted in an increase in enzyme activity with concomitant transfer of ^{32}P into the protein fraction. Subsequent incubation of the phosphorylated enzyme with millimolar Mg^{2+} revealed a decrease in activity with concomitant release of ^{32}P -phosphate from the phosphorylated protein (Trzeciak and Boyd (1974), Wallat and Kunau (1976)). Addition of bovine liver alkaline phosphatase greatly increased the dephosphorylation and deactivation of the enzyme (Beckett and Boyd (1977)). This circumstantial evidence indicated that CEH was phosphorylated and activated by cAMP-dependent protein kinase in response to the ACTH stimulus, and dephosphorylated and deactivated by the action of a Mg^{2+} -stimulated phosphoprotein phosphatase. The cAMP, Mg^{2+} and ATP concentrations required for the activation and deactivation are physiological (see Boyd and Gorban (1980)), but direct evidence of the phosphorylation and its significance in vivo requires pure enzyme.

Beckett and Boyd (1977) reported ^{32}P -incorporation into a protein with Mr 41 000 that exhibited esterase activity. However this preparation was only 57-fold purified, and a major assumption was made in comparing the results obtained on native polyacrylamide gels and SDS polyacrylamide gels. Furthermore, other proteins were phosphorylated and esterase activity (an activity stain) was measured

using 1-naphthol propionate as substrate. Naghshineh, Treadwell et al. (1978) claimed a 600-fold purification of CEH from bovine adrenal cortex, but there was no SDS/PAGE evidence to support this and specific phosphorylation of a single protein was not reported. There have been no other reports on the purification of cytosolic CEH from adrenal cortex.

To establish that an enzyme, such as CEH, undergoes physiologically significant phosphorylation-dephosphorylation, certain criteria must be satisfied (see Section 1.1). Ultimately, before all these criteria can be met, a pure preparation of enzyme must be obtained so that specific phosphorylation can be shown and correlated with changes in activity. However, it may be possible to meet these criteria if the enzyme can, at least, be identified on SDS/PAGE gels. This may be the only way of identifying and correlating changes in activity and phosphorylation in vivo in response to different metabolic situations. Further investigation of the phosphorylation process will also require pure enzyme protein. The site of the phosphorylation in CEH is unknown. Most phosphorylated proteins investigated were found to be phosphorylated on a serine or threonine residue (see Krebs and Beavo (1979)) although tyrosine residues have been found to be phosphorylated by some virus-induced protein kinase activities (Eckhart, Hutchinson and Hunter (1979), Hunter and Sefton (1980)). The amino-acid sequence about the phosphorylation site in CEH is also unknown. Sequence studies on proteins phosphorylated by cAMP-dependent protein kinase have shown that the phosphorylated serine was always 2 or 3 residues C-terminal to a pair of basic amino acids (see Krebs and Beavo (1979)). Furthermore the number and possible stoichiometry of the phosphorylation(s) and the specific biochemical effects, such as conformational changes, are unknown in CEH, and pure enzyme would be required to study these aspects.

Therefore, the urgent need in the study of adrenocorticol cytosolic CEH was the identification and purification of the enzyme protein.

There was a possibility of obtaining information about the in vivo regulation of CEH by ACTH without the purification of the enzyme. It was known that both ACTH and adrenal corticoids show a diurnal variation in their secretion in animals such as rats (Boissin, Nougier-Soulé and Assenmacher (1976)). It could be expected that adrenal CEH would also exhibit a diurnal rhythm in its activity in response to the variation in ACTH. The only evidence to date, from experiments where CEH was measured at two points in the 24 h cycle, suggested no variation in the enzyme activity (Pederson and Brownie (1979)). However more time points would be required before a clear pattern could be elucidated, or not.

The role ^{of} the cytosolic lipid droplets in ACTH-stimulated steroidogenesis had not been fully investigated. The lipid composition of the lipid droplets was well known (Moses, Davis et al. (1969), Beckett and Boyd (1975)), and the effect of diet and ACTH on the lipids in the droplets had been studied (Beckett and Boyd (1975), Trzeciak and Boyd (1974)). However, little was known about the protein fraction of the lipid droplets, although this was known to be about 2% by weight of the total (Boyd and Trzeciak (1973)). The structure and function of the protein component and its relationship to the cytosolic CEH was unstudied. As the cholesterol esters in the lipid droplets are the natural substrates for the enzyme in vivo there must be a close association between the enzyme and the lipid droplets. Accordingly it was considered essential to investigate the lipid droplet protein fraction to elucidate whether this protein exerted a physiological effect on the CEH activity.

1.3 HORMONE-SENSITIVE LIPASE (HSL)

The effect of hormones such as adrenalin, noradrenalin, glucagon and ACTH in stimulating adipose tissue lipolysis had been known for several years (Vaughan and Steinberg (1963)). The rate-limiting step in the hydrolysis of the stored triacylglycerols was known to be the triacylglycerol lipase (Vaughan and Steinberg (1965)). This lipolytic activity was found to be increased by adrenalin or cAMP (Rizack (1964)), and was therefore termed the hormone-sensitive lipase (HSL) (Vaughan, Berger and Steinberg (1964)). Subsequent studies demonstrated that partially purified HSL was phosphorylated and activated by cAMP-dependent protein kinase (Huttunen, Steinberg and Mayer (1970), Corbin, Riemann et al. (1970), Khoo, Steinberg et al. (1976)) and deactivated by phosphoprotein phosphatase (Severson, Khoo and Steinberg (1977), Khoo, Steinberg and Lee (1978), Severson and Sloan (1980)). Thus, the hormonal regulation of adipose tissue triacylglycerol lipase (HSL) appeared to involve phosphorylation and dephosphorylation of the lipase by a cAMP-dependent protein kinase and a phosphoprotein phosphatase, respectively (reviewed Steinberg (1978)). Recently, experiments with intact rat adipocytes have shown that the HSL enzyme was phosphorylated in vivo and that the phosphorylation of the enzyme and lipolysis were both increased by the addition of noradrenalin and decreased by the addition of insulin (Belfrage, Fredrikson et al. (1980), Nilsson, Stalfors et al. (1980)).

A CEH activity, that was activatable by cAMP and cofractionated with HSL through limited purification, was found in rat adipose tissue (Pittman, Khoo and Steinberg (1975)). The physiological role for a high level of activatable CEH in adipose tissue was not known as the role of cholesterol in adipose tissue was unclear, although there was a fairly large amount of cholesterol present when expressed relative to

cell protein (Farkas, Angel and Avigan (1973)). Similarly a triacylglycerol lipase activity, that was activated by ACTH in vivo, was found in rat adrenals (Gorban and Boyd (1977)). This activity extensively cofractionated with CEH activity and was activated by cAMP and ATP in vitro (Pittman and Steinberg (1977)). The physiological role of a high activity of activatable triacylglycerol lipase in adrenal cortex was not known, although there are significant amounts of triacylglycerol in the adrenal (Popjak (1944)). Both CEH and HSL activities found in adrenals and adipose tissue have similar subcellular distribution and are regulated in a similar manner. These activities also essentially copurify and show concerted binding to the two different substrates. Therefore, it was postulated that the two activities were catalysed by the same enzyme protein (Gorban and Boyd (1977), Pittman and Steinberg (1977)).

Some evidence to the contrary was also presented by Pittman and Steinberg (1977), where CEH and triacylglycerol lipase in rat adrenal appeared to show differing solubilities in ammonium sulphate and the CEH activity was selectively inhibited by chlorpyrifos oxone. However, both activities in bovine adrenocortical cytosol were found to be inactivated by chlorpyrifos oxone (Gorban (1980)). The earlier results may be re-interpreted from recent findings suggesting that bovine adrenocortical cytosol may contain two triacylglycerol lipase activities with the main activity being associated with CEH activity (Yeaman, Cook and Lee (1980)). Therefore the relationship between CEH in the adrenal and HSL in adipose tissue was unclear and required further investigation.

1.4 PHOSPHOPROTEIN PHOSPHATASES

The role of phosphoprotein phosphatases in the regulation of enzyme activity by reversible phosphorylation reactions is less well known than

the role of protein kinases (see Krebs and Beavo (1979)). Much work has been performed in recent years to rectify this situation. However this area has proved to be even more complicated and confusing than the protein kinase field. Most work on phosphoprotein phosphatases has centred on glycogen metabolism using tissues such as liver, skeletal muscle and cardiac muscle with phosphorylase a, or other glycogen metabolising enzymes, as substrate (see Fischer and Brautigan (1982)).

It now appears that a major problem in elucidating the role of phosphoprotein phosphatases will be associated with the purification of the physiological forms of the enzyme. In many studies, phosphoprotein phosphatase purifications revealed multiple molecular weight forms of the enzymes from very high (300 000-200 000) to low (30 000)(Kalala, Goris and Merlevede (1973), Kato, Kobayashi and Sato (1974), Kobayashi and Kato (1977), Antoniow, Nimmo et al. (1977)). There was a body of evidence suggesting that phosphorylase phosphatase had a molecular weight of approximately 260 000 (Killilea, Mellgren et al. (1979), Mellgren, Aylward et al. (1979)). It was also discovered that the high molecular weight forms could be dissociated by treatment with ethanol to yield a phosphoprotein phosphatase with Mr 30 000-35 000 (Brandt, Killilea and Lee (1974)). Several other harsh, denaturing treatments could also lead to the production of the low molecular weight phosphoprotein phosphatase, but increasing salt concentration could also dissociate the Mr 260 000 phosphoprotein phosphatase (Khandelwal, Zinman and Ng (1980)). The Mr 35 000 phosphoprotein phosphatase was found to have low substrate specificity and generally no ion requirements and has been termed the multifunctional phosphoprotein phosphatase (Krebs and Beavo (1979)). It was postulated that the Mr 35 000 phosphoprotein phosphatase was the catalytic subunit of many of the high molecular weight, physiological forms of phosphoprotein

phosphatase, with the other subunits conferring substrate specificities, ion requirements and other regulatory controls on the activity of the catalytic subunit (Brandt, Capulong and Lee (1975), Kobayashi and Kato (1977)). There have been studies investigating the structure of the physiological, high molecular weight forms, but little is known about the specific functions of the other subunits (Tamura and Tsuiki (1980), Tamura, Kikuchi et al. (1980), Imazu, Imaoka et al. (1981)).

The dissociation of the high molecular weight forms to release the Mr 35 000 subunit has been shown in liver (Killilea, Brandt et al. (1976)), heart (Li, Hsiao and Chan (1978)), adrenal (Li (1979)) and various other tissues including skeletal muscle, brain, kidney and uterus (Li and Chan (1981)). However, not all phosphoprotein phosphatases may contain this catalytic subunit, as one group has purified, from skeletal muscle, a catalytic subunit with Mr 70 000 (F_c) which was activated by a further subunit with Mr 50 000 (F_a) (Yang, Vandenheede et al. (1980b), Vandenheede, Yang et al. (1980)). Although this activation required ATP-Mg²⁺, no phosphorylation was involved in the reaction (Yang, Vandenheede et al. (1980a)). This enzyme was first discovered in adrenal cortex (Merlevede and Riley (1966)) and has since been found in liver, skeletal and cardiac muscle from rat and rabbit (Yang, Vandenheede et al. (1980a)). The relationship of this phosphoprotein phosphatase to the other phosphoprotein phosphatases is not fully understood (Vandenheede, Yang and Merlevede (1981), Stewart, Hemmings et al. (1981)).

Phosphoprotein phosphatases have been purified from adrenal cortex resulting in high molecular weight forms (Kalala, Goris and Merlevede (1973), Ullman and Perlman (1975)), or, after ethanol treatment, the Mr 35 000 subunit (Li (1979)). These phosphoprotein phosphatases were active towards enzymes of glycogen metabolism or synthetic phosphoprotein

substrates such as phosphoprotamine, but no phosphoprotein phosphatase purified from adrenal cortex had been studied with respect to its role in the deactivation of CEH. It was known that incubation of CEH with millimolar Mg^{2+} resulted in a deactivation of the enzyme (Trzeciak and Boyd (1973)), and that adrenal phosphoprotein phosphatases were stimulated by Mg^{2+} or Mn^{2+} (Merlevede and Riley (1966), Ullman and Perlman (1975)). Therefore it was postulated that the deactivation of CEH was catalysed by a phosphoprotein phosphatase, and this was confirmed when exogenous bovine liver alkaline phosphatase was shown to deactivate CEH (Beckett and Boyd (1977)).

Partially purified phosphoprotein phosphatase from chicken adipose tissue was found to deactivate HSL from the same tissue in a Mg^{2+} -dependent reaction (Severson, Khoo and Steinberg (1977)). Furthermore, a low molecular weight phosphoprotein phosphatase had been purified from rat adipose tissue and this was also found to deactivate HSL (Severson and Sloan (1980)). However, there had been no investigation of the role of endogenous, adrenocortical cytosolic phosphoprotein phosphatase in the regulation of cytosolic CEH activity in the adrenal cortex.

1.5 HEAT-STABLE INHIBITOR PROTEINS OF cAMP-DEPENDENT PROTEIN KINASE AND PHOSPHOPROTEIN PHOSPHATASE

It was known that a heat-stable, trypsin-labile protein capable of inhibiting cAMP-dependent protein kinase was present in many tissues including adrenal cortex (Walsh, Ashby et al. (1971), Krebs and Beavo (1979), Cochet, Job and Chambaz (1977b)). The inhibitor has a molecular weight of 11 300 and binds to the catalytic subunit with a K_i of about $2 \times 10^{-9} M$ and so blocks the activity (Krebs and Beavo (1979)). The physiological role of this protein is not known but it may inhibit the catalytically active protein kinase when cAMP concentrations are low.

A second inhibitor of cAMP-dependent protein kinase, isolated from rat testis, has a molecular weight of 21 600 and also inhibits cAMP phosphodiesterase. A third inhibitor ("type II inhibitor") from brain, inhibits protein phosphorylation catalysed by several protein kinases including cAMP-dependent protein kinase (see Krebs and Beavo (1979)). A heat-stable inhibitor protein of the cyclic nucleotide-independent protein kinase that can use either ATP or GTP as a phosphate donor (CKG) has been found in adrenal cortex (Job, Cochet et al. (1979)).

Heat-stable protein inhibitors of phosphoprotein phosphatase were discovered in rabbit liver (Brandt, Lee and Killilea (1975)), skeletal muscle (Huang and Glinnsmann (1975)) and later in various tissues including adrenal cortex (Huang, Tao and Glinnsmann (1977)). Two types of inhibitor are known to exist. Inhibitor - 1, purified to homogeneity from skeletal muscle, had a molecular weight of about 20 000-26 000 and was found to be phosphorylated by cAMP-dependent protein kinase, and only in this form did it inhibit phosphoprotein phosphatase (Nimmo and Cohen (1978a&b)). It was later found to be dephosphorylated by phosphoprotein phosphatase and it did not inhibit its own dephosphorylation (Foulkes, Jefferson and Cohen (1980)). In vivo experiments have shown that the degree of phosphorylation of inhibitor - 1 was modulated by administration of adrenalin in skeletal muscle (Tao, Huang et al. (1978), Foulkes and Cohen (1979)) and adipose tissue (Belsham and Denton (1980)) and insulin in skeletal muscle and adipose tissue (Foulkes, Jefferson and Cohen (1980), Belsham and Denton (1980)). Inhibitor - 2 has also been purified to homogeneity, has a molecular weight of about 33 000 in rabbit skeletal muscle and is not modulated by phosphorylation (Huang and Glinnsmann (1976), Foulkes and Cohen (1980)). A physiological role for inhibitor - 2 was postulated recently when it was discovered to be an essential protein for the full activation of the F_c phosphoprotein

phosphatase by the F_a activating protein (Vandenheede, Goris et al. (1981), Yang, Vandenheede et al. (1981)).

It was thought that the heat-stable inhibitors may be subunits of the high molecular weight phosphoprotein phosphatases, that are released by heat treatment, but destroyed by ethanol treatment. A recent study, however, has provided evidence that the activity of the Mr 35 000 catalytic subunit was not related to destruction of the inhibitor protein (Jett and Hers (1981)). A heat-stable protein, that prevented the deactivation of HSL by a Mg^{2+} -dependent phosphoprotein phosphatase was found in chicken adipose tissue (Severson and Sloan (1977)). This inhibitor was isolated from chicken adipose tissue by a similar procedure to that used for the isolation of bovine adrenal phosphoprotein phosphatase inhibitors (Huang, Tao and Glinsmann (1977)). However it is not known whether the adipose tissue phosphoprotein phosphatase inhibitor is phosphorylatable.

The heat-stable protein inhibitor of cAMP-dependent protein kinase, phosphoprotein phosphatase inhibitor - 1 and inhibitor - 2 have all been demonstrated in adrenal cortex (Cochet, Job and Chambaz (1977b), Huang, Tao and Glinsmann (1977)). The existence of cAMP-dependent protein kinase, phosphoprotein phosphatase and their respective heat-stable inhibitor proteins in adrenal cortex led Gorban and Boyd (1980) to propose an integrated control system for cytosolic CEH as shown in Fig. 1.3. In this system a rise in intracellular cAMP and the subsequent activation of cAMP-dependent protein kinase would not only directly activate CEH, but also "switch off" the deactivation of the enzyme by phosphoprotein phosphatase through the phosphorylation and activation of phosphoprotein phosphatase inhibitor - 1. There had been no investigation of the role of phosphoprotein phosphatase or any of the heat-stable inhibitor proteins in adrenocortical cytosol and so this postulated system was yet to be proved.

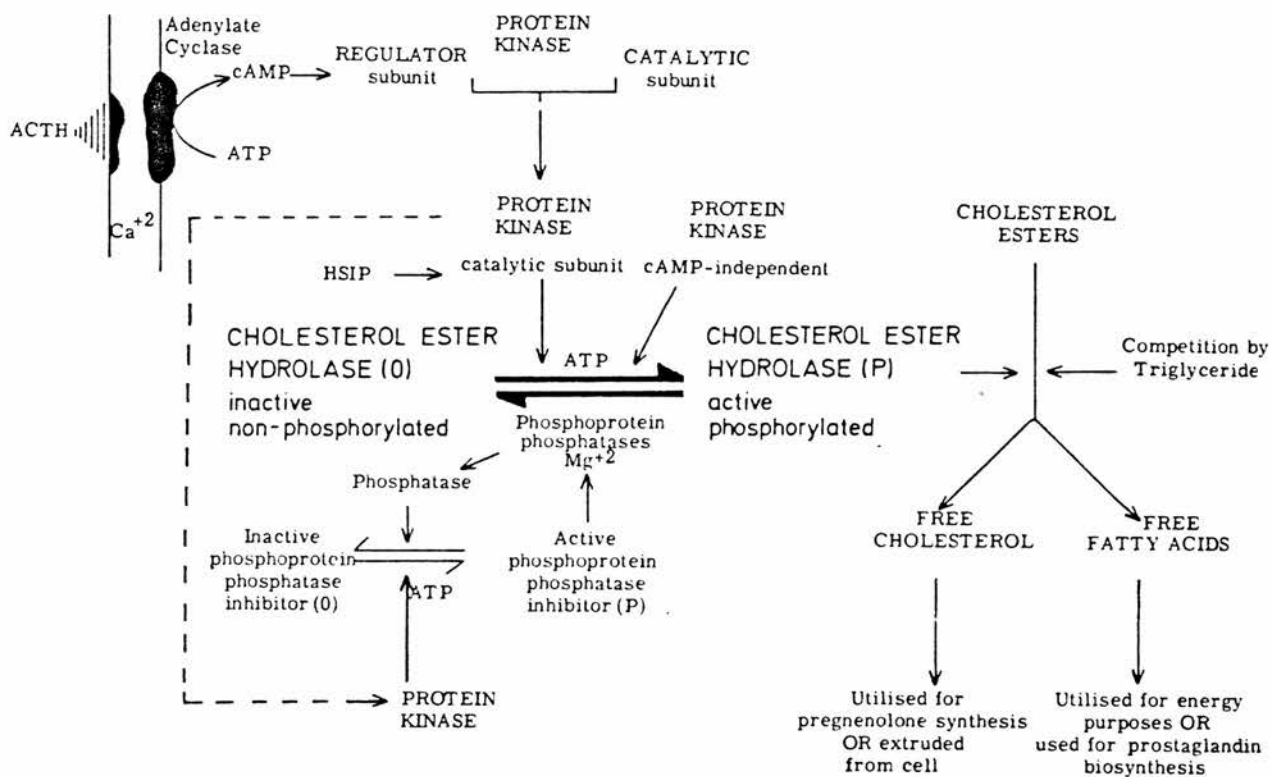


Fig. 1.3. Postulated control system for the regulation of CEH activity in adrenal cortex.

(Taken from Gorban and Boyd (1980)).

1.6 THE AIMS OF THE PRESENT STUDY

The urgent need in the study of adrenocortical cytosolic CEH is to obtain a pure preparation of the enzyme. When this is achieved there are three main areas to be investigated.

Firstly the criteria formulated by Krebs (1972) and later modified by Krebs and Beavo (1979) must be studied to establish whether the phosphorylation-dephosphorylation is physiologically significant and occurs in vivo. Secondly, the phosphorylation process must be studied in detail to elucidate the site(s) of phosphorylation, the amino-acid sequence about the site(s), the number and stoichiometry of the site(s), the order of phosphorylation of different sites and the precise biochemical and molecular effects of the phosphorylation. Finally the regulation of the enzyme must be investigated to establish whether the posulated control mechanism (see Fig. 1.3) operates in vivo.

Considering these three areas of investigation as the ultimate aim, it was hoped that the following lines of research might resolve several of the main problems:

1. To further purify the cytosolic CEH from bovine adrenocortical cytosol using three major protein separation techniques, not previously attempted with this enzyme; detergent solubilisation, hydrophobic chromatography and affinity chromatography.
2. To identify the CEH enzyme protein in bovine adrenocortical cytosol, and investigate its relationship with the HSL of adipose tissue cytosol.
3. To purify a phosphoprotein phosphatase from bovine adrenocortical cytosol and investigate its role in the dephosphorylation and deactivation of bovine adrenocortical cytosolic CEH in vitro.
4. To investigate the regulation of CEH in rat adrenal by ACTH in vivo.
5. To identify the protein components of rat adrenal and bovine adrenocortical lipid droplets, and investigate their relationship to the cytosolic CEH.

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2.1 MATERIALS

Cholesterol oleate (99% pure), glycerol trioleate (98% pure), oleic acid, essentially fatty acid-free bovine serum albumin, cholic acid, cGMP, type II (bovine heart) cAMP-dependent protein kinase, catalytic subunit of cAMP-dependent protein kinase, PAGE blue 83 and Coomassie brilliant blue G were all obtained from Sigma (London) Chemical Co. Ltd. (Poole).

Cholesterol $[1-^{14}\text{C}]$ oleate (approximately 50 mCi.mmol^{-1}), glycerol tri- $[1-^{14}\text{C}]$ oleate (48 mCi.mmol^{-1}), $[^{32}\text{P}]$ orthophosphate (1 mCi.ml^{-1} , carrier free), $[^{125}\text{I}]$ iodide (100 mCi.ml^{-1} , carrier free) and $[1,3-^3\text{H}]$ DFP (6 Ci.mmol^{-1}) were all obtained from The Radiochemical Centre Ltd., Amersham.

Phosphoglycerate kinase/glyceraldehyde 3-phosphate dehydrogenase suspension from yeast/rabbit muscle, ATP and cAMP were obtained from Boehringer Corporation (London) Ltd., Lewes.

ACTH (Acthar) was a product of Armour Pharmaceutical Co. Ltd. (Eastbourne).

Dexamethasone sodium phosphate (Decadron) was a product of Merck Sharp Dohme Ltd. (Hoddesdon).

NCS tissue solubiliser was obtained from G.D. Searle and Co. Ltd. (High Wycombe).

Shaltiel hydrophobic chromatography test kits and crystalline bovine serum albumin were obtained from Miles Laboratories, Slough.

Sepharose CL-4B, Sephadex G-25, Sephadex G-100, Sephacryl S-200, DEAE-Sephadex A-50, QAE-Sephadex A-25 and Cibacron blue - Sepharose 4B were obtained from Pharmacia (Great Britain) Ltd., Hounslow.

Hydroxylapatite (Bio-Gel HTP) was a product of Bio Rad Laboratories Ltd. (Watford).

DEAE-Cellulose (DE52) was a product of Whatman Ltd. (Maidstone, Kent). Ultragel Aca34 was obtained from LKB Instruments Ltd. (South Croyden, Surrey).

C₁₃ E₁₂ detergent (Berol 038) was a gift from Dr. Per Belfrage, Department of Physiological Chemistry, University of Lund, Lund, Sweden.

All other common reagents and chemicals were obtained from Sigma (London) Chemical Co. Ltd., BDH (Poole, U.K.) or Koch-Light (Bucks, U.K.) and were of analytical grade.

2.2 BOVINE ADRENAL CORTEX

Bovine adrenals were obtained from the local abattoir and transported to the laboratory on ice, arriving there within one hour of the animals' death. The adrenal glands were trimmed of adhering fat, cut longitudinally and the medulla removed and discarded. The cortex was scraped from the capsule and immersed in ice cold 0.25 M sucrose containing 1 mM EDTA and 10 mM potassium phosphate buffer pH 7.4. When phosphoprotein phosphatase was to be prepared from the adrenal cortex, 10 mM Tris-HCl buffer pH 7.4 at 4°C replaced the potassium phosphate buffer. A 20% (w/v) homogenate was prepared using a Potter-Elvehjem glass/teflon homogeniser. The homogenate was centrifuged and the 105 000 x g supernatant prepared as described in Section 2.4.

2.3 ANIMALS AND ANIMAL PRETREATMENT

For experiments on the in vivo variation of CEH the experimental animals were adult female rats (150-200 g) of the Wistar strain bred in the Faculty Animal Facility, Hugh Robson Building, University of Edinburgh. They were maintained on a stock diet consisting of 25% skimmed milk powder, 5% dried yeast and 70% wholemeal flour. They were given water ad libitum. The animals were divided randomly into two groups. One group was kept in the normal light dark cycle (light 08.00-20.00 h), while the other group was maintained in the reversed cycle (dark 08.00-20.00 h) for at least two weeks, to become fully adapted.

2.3.1 ACTH administration

Adult female rats (150-200 g) of the Wistar strain were divided randomly into two groups. One group received subcutaneous injections of 8 i.u. ACTH (Acthar Coricotrophin (sterile)) dissolved in 0.2 ml saline, 30 min before sacrifice, while the control group received 0.2 ml saline. The animals were killed by decapitation and blood collected from the neck for serum corticosterone measurements as described in Section 2.24. The adrenals were excised, decapsulated and homogenised in 0.25 M sucrose containing 1 mM EDTA and 10 mM potassium phosphate buffer pH 7.4. The homogenate was centrifuged and the 105 000 x g supernatant prepared as described in Section 2.4.

2.3.2 Corticoid administration

Adult female rats (150-200 g) of the Wistar strain were divided randomly into two groups. One group received subcutaneous injections of 500 µg of corticoid in a total volume of 0.2 ml of the appropriate carrier at 72 h and 24 h prior to killing. The control group received 0.2 ml of carrier. The animals were killed by decapitation. Blood was collected and adrenals excised and processed as described in Section 2.3.1.

2.4 PREPARATION OF 105 000 x g SUPERNATANT

The 105 000 x g supernatant from rat adrenal and bovine adrenal cortex was prepared from the homogenate of the appropriate tissue according to the procedure shown in Fig. 2.1.

The homogenate was centrifuged at 10 000 x g at 4°C for 10 min to remove cell debris, nuclei, mitochondria, lysosomes and the floating lipid layer. The 10 000 x g infranatant was centrifuged at 105 000 x g for 1 hour at 4°C. The lipid droplets were removed by aspiration and the clear infranatant was pooled, discarding the microsomes. The 105 000 x g infranatant was used as the enzyme source for the purification of CEH and was referred to as the tissue cytosol.

Tissue homogenate was prepared by homogenising the tissue in 10 mM potassium phosphate (or Tris-HCl) buffer pH 7.4 containing 0.25 M sucrose and 1 mM EDTA in a motorised Potter-Elvehjem glass-teflon homogeniser

The 20% (w/v) homogenate was centrifuged at 10 000 x g for 10 min at 4°C in a fixed angle rotor, Beckman J2-21 centrifuge

Cell debris, nuclei
mitochondria and
lysosomes discarded

Infranatant

Lipid droplets
aspirated off

Centrifuged at 105 000 x g for 1 hour at 4°C in a Beckman ultracentrifuge

Microsomes and light
mitochondria discarded

Infranatant

Lipid droplets
aspirated off

TISSUE CYTOSOL

Pooled for enzymic assay and/or further purification

Fig. 2.1. The flow diagram for the preparation of the soluble tissue fraction (cytosol) from rat adrenal and bovine adrenal cortex.

2.5 PREPARATION OF THE LIPID DROPLET FRACTION

The lipid droplet fraction from rat adrenal and bovine adrenal cortex homogenates were prepared by harvesting the floating lipid layer obtained at the 105 000 x g centrifugation step in the preparation of tissue cytosol. The lipid layer was collected using the apparatus shown in Fig. 2.2, which enabled the lipid droplets to be aspirated off the surface of the liquid by suction. This minimised the contamination of the lipid droplets by the infranatant material.

The lipid droplets were washed twice with 10 volumes ice-cold 0.25 M sucrose containing 1 mM EDTA and 10 mM potassium phosphate buffer pH 7.4 by centrifugation at 105 000 x g for 90 min at 4°C. The washed lipid droplets were dialysed overnight at 4°C against 500 volumes 10 mM potassium phosphate buffer pH 7.4.

2.6 PREPARATION OF EMULSIFIED CHOLESTEROL $[1-^{14}\text{C}]$ OLEATE AND GLYCEROL TRI- $[1-^{14}\text{C}]$ OLEATE

The emulsified substrate used for the assay of CEH and triacylglycerol hydrolase was prepared according to Khoo, Steinberg et al. (1976) with some modifications.

A known quantity of cholesterol $[1-^{14}\text{C}]$ oleate in toluene was added to unlabelled cholesterol oleate dissolved in the same solvent. The toluene was evaporated by a stream of nitrogen, and the dried pellet was dissolved in warm absolute ethanol. This solution was injected in small batches into an ice-cold mixture of fatty acid-free bovine serum albumin, EDTA and potassium phosphate buffer pH 7.4. The mixture was left to stir for 15 min before storing at 4°C. Before use the solution was stirred for 2-3 min. The substrate was prepared in batches of 20 ml and used within 2 weeks of preparation. The final constitution of the mixture was: 500 μM cholesterol $[1-^{14}\text{C}]$ oleate (specific activity 1.0-2.0 $\mu\text{Ci} \cdot \mu\text{mol}^{-1}$), 5% (w/v) fatty acid-free

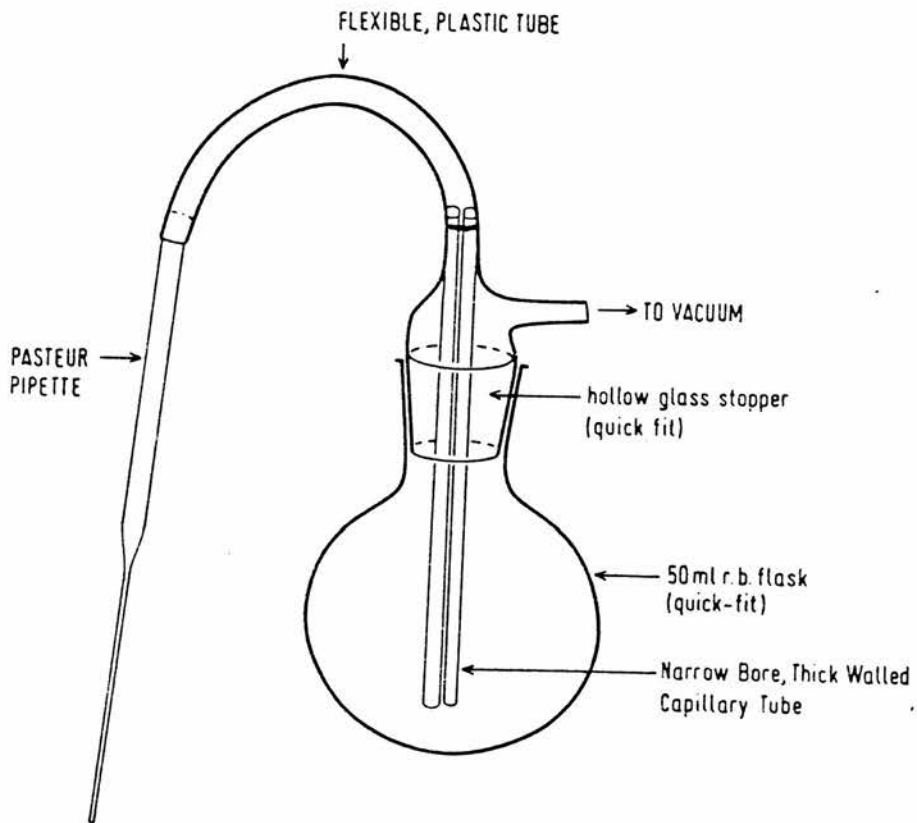


Fig. 2.2. Apparatus for the collection of lipid droplet fractions.

The apparatus was used to aspirate off and collect the floating lipid droplet fraction obtained at the 105 000 x g centrifugation step in the preparation of tissue cytosol.

bovine serum albumin, 5% (v/v) ethanol, 10 mM EDTA and 100 mM potassium phosphate buffer. The final pH was 7.2-7.4.

Glycerol tri- $[1-^{14}\text{C}]$ oleate was prepared for use in the same way and in the same concentration of 500 μM .

2.7 THE ASSAY OF CEH AND TRIACYLGLYCEROL HYDROLASE

To an enzyme solution in potassium phosphate (or Tris-HCl) buffer pH 7.4 in a final volume of 0.2 ml was added 0.2 ml of emulsified cholesterol $[1-^{14}\text{C}]$ oleate or glycerol tri- $[1-^{14}\text{C}]$ oleate substrate. The incubation was carried out at 37°C for 30-45 min, as appropriate. Final concentrations in the incubation mixture were: 250 μM cholesterol $[1-^{14}\text{C}]$ oleate (100 nmol, 0.1 μCi), 2.5% (w/v) (0.38 mM) bovine serum albumin, 2.5% (v/v) ethanol, 5 mM EDTA and 50 mM potassium phosphate buffer. The enzyme protein concentration did not exceed 0.5 mg ml^{-1} per incubation and the final pH of the incubation was routinely checked and found to be 7.1-7.4. Blank incubations, omitting the enzyme were always run in parallel.

The enzymic hydrolysis of the cholesterol ester substrate was terminated by the addition of 1.5 ml fatty acid extraction mixture (Khoo and Steinberg (1975)) which contained chloroform/methanol/toluene (2:2.4:1, v/v) with 0.29 mM unlabelled oleic acid as a carrier. After the addition of 50 μl 1.0 M NaOH the tubes were vortexed for 15 s and centrifuged at $1500 \times g$ for 20 min at room temperature. A 500 μl sample from the upper aqueous phase (1 ml total) was taken into 10 ml Triton X-100/toluene scintillation cocktail (0.33 l Triton X-100, 0.67 l toluene containing 4 g l^{-1} 2'5'-diphenyloxazole (PPO) and 0.03 g l^{-1} 1'4'-bis-5-phenyloxazyl-2-benzene (POPOP)).

The estimation of $[1-^{14}\text{C}]$ oleic acid was carried out in a Packard Tri-Carb liquid scintillation spectrometer. Recovery of $[1-^{14}\text{C}]$ oleic acid from the incubation by the extraction was found to be 76-80%

(Gorban (1980)). Counting efficiency for $[1-^{14}\text{C}]$ oleic acid was 80% and quenching was monitored by an external standard.

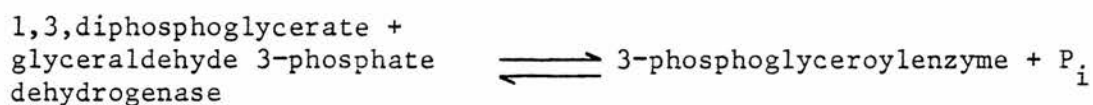
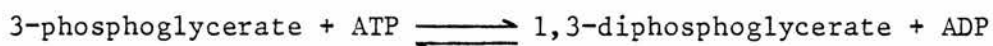
In some assays the amount of substrate was reduced to 0.1 ml in a total volume of either 0.2 ml or 0.4 ml giving final cholesterol $[1-^{14}\text{C}]$ oleate concentrations of 250 μM and 125 μM , respectively. In the former case 250 μl 0.2 M NaOH was added to the tubes after the fatty acid extraction mixture to keep the solvent ratios and NaOH concentration identical to the basic assay. The effect of enzyme protein and incubation time on the CEH assay had been investigated in a previous study (Gorban (1980)).

2.8 IN VITRO ACTIVATION AND DEACTIVATION OF CEH

To an enzyme solution in potassium phosphate (or Tris-HCl) buffer pH 7.4 containing a maximum of 250 μg protein in a total volume of 100 μl was added 100 μl of a mixture of magnesium chloride, ATP and cAMP in Tris-HCl buffer pH 7.4 at 37°C. The final concentrations in the incubation were: 5 mM magnesium chloride, 5 mM ATP, 20 μM cAMP and 20 mM Tris-HCl. These were the optimum concentrations of ATP and cAMP for the in vitro activation of cytosolic CEH as determined by Gorban (1980). The CEH activation reaction was carried out for 10 min at 37°C after which time it was terminated by the addition of substrate emulsion containing excess EDTA. Deactivation of the enzyme was carried out in a similar way to the activation but with the omission of ATP and cAMP. The incubation mixture for the basal activity of the enzyme contained no magnesium chloride, no ATP and no cAMP.

2.9 PREPARATION OF $[\gamma-^{32}\text{P}]$ ATP

$[\gamma-^{32}\text{P}]$ ATP was prepared by a modification of the procedure of Glynn and Chappell (1964). This method utilises the exchange reaction between ATP and $[^{32}\text{P}]$ orthophosphate that occurs in the presence of phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase and suitable substrates.



The reaction mixture contained 40 μl 1.0 M magnesium acetate, 100 μl 0.1 M ATP, 10 μl 0.1 M EDTA, 10 μl 2-mercaptoethanol, 40 μl 0.2 M 3-phosphoglycerate and 775 μl 1.0 M Tris-HCl buffer pH 8.0 at room temperature. The reaction was initiated by the addition of 2 ml carrier-free $\left[^{32}\text{P} \right]$ orthophosphate (2 mCi) in dilute HCl followed by 25 μl of phosphoglycerate kinase/glyceraldehyde 3-phosphate dehydrogenase enzyme mixture (Boehringer Corporation (London) Ltd., Lewes). The incubation was for 3 h at room temperature. For certain experiments the specific activity of the $\left[\gamma\text{-}^{32}\text{P} \right]$ ATP was increased 2.5 fold by reducing the amount of ATP added to 4 μmoles in 100 μl .

To calculate the yield of the reaction, 5 μl of the mixture was added to 5 ml distilled water and the solution vortexed. Samples of 10 μl of this solution were added to 10 ml Triton X-100/toluene scintillation cocktail and the radioactivity estimated in a Packard Tri-Carb liquid scintillation spectrometer. Excess activated charcoal was added to the sample solution which was vortexed and centrifuged at 1000 x g. Samples of 10 μl of the supernatant were taken and counted as above. The yield of $\left[\gamma\text{-}^{32}\text{P} \right]$ ATP was calculated from $100 - \left(\frac{\text{cpm after charcoal}}{\text{cpm before charcoal}} \times 100 \right)$ and was routinely 98-99% when the specific activity was 0.2 mCi. μmol^{-1} (400 cpm.pmol $^{-1}$) and 94% when the specific activity was 0.5 mCi. μmol^{-1} (1000 cpm.pmol $^{-1}$).

2.10 PREPARATION OF ^{32}P -LABELLED PHOSPHOHISTONE

^{32}P -labelled phosphohistone was prepared by a modification of the basic method of Meisler and Langan (1969).

The concentrations in 3 ml final incubation mixture were 670 μM $[\gamma\text{-}^{32}\text{P}]$ ATP ($0.2 \text{ mCi} \cdot \mu\text{mol}^{-1}$), 2.2 mgml^{-1} calf thymus histone, 8 mM magnesium acetate, 2 μM cAMP, 0.33 mgml^{-1} cAMP-dependent protein kinase (type II from bovine heart) and 50 mM TES buffer pH 7.0. The reaction was initiated by the addition of the cAMP-dependent protein kinase and the incubation continued for 4 h at 30°C .

The reaction was terminated by the addition of ice-cold 25% (w/v) TCA to give a final TCA concentration of 2.5% (w/v). The solution was centrifuged at $1000 \times g$ for 10 min and the precipitated protein kinase removed. To the clear supernatant was added ice-cold 50% (w/v) TCA to give a final TCA concentration of 25% (w/v). The mixture was centrifuged at $1000 \times g$ for 10 min and the supernatant discarded. The pellet was redissolved in distilled water, reprecipitated with ice-cold 25% (w/v) TCA and the pellet collected by centrifugation as before. The pellet was washed twice with ethanol/diethyl ether (1:4, v/v), then twice with ethanol/diethyl ether (1:4, v/v) containing 0.1 M HCl. The product was dried overnight by vacuum desiccation, redissolved in 5 ml 50 mM TES buffer pH 7.0 and divided into 1 ml portions for subsequent use.

Samples of 20 μl were taken and ^{32}P incorporation into protein was measured (see Section 2.11). The preparation was routinely found to be approximately 97% ^{32}P -labelled phosphoprotein with a specific activity of $4\text{--}5 \text{ } \mu\text{Ci} \cdot \text{mg protein}^{-1}$ indicating about 8% incorporation of ^{32}P into protein.

2.11 ^{32}P -LABELLED PHOSPHOPROTEIN ASSAY

The assay of ^{32}P -labelled phosphoprotein was carried out similar to the method of Walsh, Perkins et al. (1971).

To 0.4 ml ^{32}P -labelled phosphoprotein was added 0.1 ml bovine serum albumin (12.5 mgml^{-1}) followed by 0.5 ml ice-cold 10% (w/v) TCA. The mixture was left for 10 min at 4°C and then centrifuged at $1500 \times g$

for 10 min at 4°C. An aliquot of the supernatant was taken into 10 ml Triton X-100/toluene scintillation cocktail (see Section 2.7) and the non-TCA precipitated radioactivity estimated in a Packard Tri-Carb liquid scintillation spectrometer. The remaining supernatant was decanted and the pellet redissolved in 50 µl 0.1 M NaOH, reprecipitated quickly with 1 ml ice-cold 10% (w/v) TCA, left for 10 min at 4°C and recentrifuged. The pellet was washed twice with 1 ml ice-cold 5% (w/v) TCA, dissolved in 50 µl 23 M formic acid and decanted into 5 ml Triton X-100/toluene scintillation cocktail. The tube was then washed with a further 5 ml scintillation cocktail to ensure maximum recovery.

2.12 PROTEIN KINASE ASSAY

Protein kinase activity was assayed by a modification of the method of Walsh, Perkins and Krebs (1968).

The assay mixture contained 125 mM 2-glycerophosphate (disodium salt), 6 mgml⁻¹ calf thymus histone, 50 mM sodium fluoride, 5 mM theophylline, 9 mM magnesium acetate and 0.75 mM EGTA. To this was added cAMP, cGMP or calcium chloride up to final concentrations of 250 µM, 250 µM and 2 mM, respectively. A portion of assay mixture, without these additions, was kept for control incubations and all four solutions were adjusted to pH 6.0 with HCl.

To 40 µl of the assay mixture (with and without additions) was added 10 µl 1.2 mM $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP (0.2 mCi.µmol⁻¹) and 50 µl of protein solution or buffer. Incubation was for 10 min at 30°C and the assay was terminated by the addition of 0.1 ml 12.5 mgml⁻¹ bovine serum albumin and 0.5 ml ice-cold 10% (w/v) TCA. The ³²P-labelled phosphoprotein was determined as described in Section 2.11.

2.13 PHOSPHOPROTEIN PHOSPHATASE ASSAY

To 20 µl ³²P-labelled phosphohistone (0.1 µCi, 20 µg) or an appropriate amount of other ³²P-labelled phosphoprotein was added 50 µl

phosphoprotein phosphatase preparation (see appropriate sections for description) and the volume made up to 0.4 ml with buffer. The incubation period was 10 min at 37°C. The reaction was terminated and ^{32}P -orthophosphate release and ^{32}P -labelled phosphoprotein determined as described in Section 2.11.

2.14 ALKALINE PHOSPHATASE ASSAY

Alkaline phosphatase activity was assayed essentially as described by Li (1979) with some modification.

To 0.25 ml phosphatase preparation (see Section 7) was added 0.25 ml assay reagent which contained 20 mM p-nitrophenyl phosphate, 20 mM magnesium chloride, 1 mM DTT and 50 mM Tris-HCl buffer pH 8.6 at 30°C. The incubation period was 20 min at 30°C and the reaction was terminated by the addition of 0.5 ml 1.0 M sodium carbonate.

The phosphatase activity was measured spectrophotometrically by the absorbance at 410 nm of the p-nitrophenol released from p-nitrophenyl phosphate using a control, omitting enzyme, as a blank (absorption coefficient for p-nitrophenolate ion $1.75 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

2.15 PREPARATION OF HIGH-DENSITY LIPOPROTEIN (HDL) FROM HUMAN SERUM

High-density lipoprotein (HDL) was prepared from human serum by phosphotungstate precipitation (Burstein and Scholnick (1973)) and density gradient ultracentrifugation.

To 1 litre human serum was added 100 ml 4% (w/v) sodium phosphotungstate (adjusted to pH 7.0 with NaOH) and 25 ml 2.0 M magnesium chloride. The material was immediately centrifuged at 6000 x g for 10 min and the precipitated very low density lipoprotein (VLDL) and low density lipoprotein (LDL) discarded. To the supernatant was added 900 ml 4% (w/v) sodium phosphotungstate and the precipitated γ -globulins removed by immediate centrifugation at 6000 x g for 10 min. The supernatant had 175 ml 2.0 M magnesium chloride added to it and

was left for 2 h at room temperature before centrifugation at 20 000 x g for 20 min. The precipitate was washed with 500 ml of a solution containing 0.4% (w/v) sodium phosphotungstate, 0.1 M magnesium chloride and 1% (w/v) sodium chloride and centrifuged at 6000 x g for 10 min. The precipitate was resuspended in 70 ml 1% (w/v) sodium chloride and solubilisation achieved by the dropwise addition of 10% (w/v) sodium carbonate, with stirring.

To the concentrated, neutral solution of HDL was added potassium bromide up to a final density of 1.21 and the resultant solution was centrifuged at 100 000 x g for 20 h at 4°C. The floating layer of HDL was removed using a Pasteur pipette.

2.16 PREPARATION OF APOLIPOPROTEIN A-I FROM HUMAN HDL

HDL was delipidated according to Scanu (1966). The delipidated protein (approximately 30 mg) was dissolved in 15 ml 10 mM Tris-HCl buffer pH 8.0 at 4°C containing 6 M urea and 1 mM EDTA and subjected to chromatography on a Sephacryl S-200 column (3 x 50 cm) equilibrated in the same buffer. The fractions were assayed for apolipoprotein A-I by SDS/PAGE (see Section 2.18) and the pooled fractions were applied to a Sephadex G-100 column (3 x 75 cm) equilibrated in the same buffer. The fractions containing apolipoprotein A-I were pooled, but were still contaminated by albumin and several other proteins (Fig. 2.3a).

The main contaminant was albumin and it was expected that it could be removed by utilising its multi-functional binding properties, in particular its affinity for dyes. The pooled sample was dialysed against 20 mM ammonium bicarbonate containing 0.1 M sodium chloride, and loaded onto a Cibacron Blue-Sepharose 4B column (3 x 15 cm) equilibrated with the buffer. The fractions were assayed by SDS/PAGE as before, and both albumin and apolipoprotein A-I bound to the column. This was expected as this column had been used in purifications of both

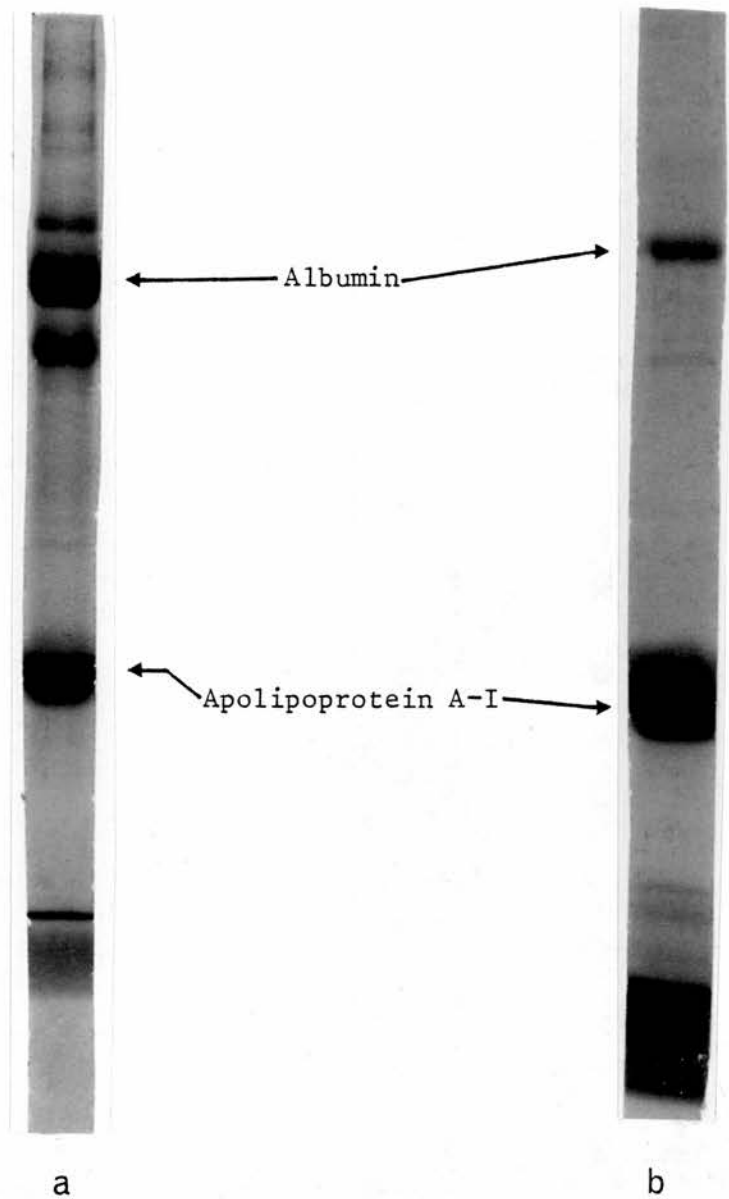


Fig. 2.3. Purification of apolipoprotein A-I from delipidated human HDL. Samples of approximately 100 μ g protein from the pooled fractions after a) Sephadex G-100 chromatography and b) Cibacron Blue - Sepharose 4B chromatography were subjected to SDS/PAGE in 10% gels as described in Section 2.18.

albumin and lipoproteins (see Gianazza and Arnaud (1982)). The other contaminants were eluted at this stage. The column was washed with several volumes 2.0 M sodium chloride, in the column buffer, and a portion of the albumin was eluted. Finally 6 M urea was included in the buffer eluting the remaining albumin in a broad peak. However, apolipoprotein A-I was eluted in a sharp peak and subsequent analysis of the pooled fractions by SDS/PAGE indicated only minor contamination by albumin (Fig. 2.3b). The apolipoprotein A-I was extensively dialysed against 20 mM ammonium hydrogen carbonate, lyophilised and stored at -20°C for subsequent use.

2.17 ^{125}I -LABELLING OF APOLIPOPROTEIN A-I

Apolipoprotein A-I was labelled with $\left[^{125}\text{I}\right]$ iodide by a method essentially as described by Bolton (1977).

Apolipoprotein A-I (1 mg in 0.5 ml) in 50 mM sodium phosphate buffer pH 7.4 was incubated with 0.25 ml 40 mM chloramine-T (in the above buffer) and 10 μl $\left[^{125}\text{I}\right]$ iodide for 10 min at 0°C . The reaction was terminated by the addition of 0.25 ml 100 mM sodium metabisulphite in the above buffer and the mixture desalted on a Sephadex G-25 column (1 x 16 cm) equilibrated in the same buffer.

The incorporation was calculated to be 90% and the specific activity of the peak fraction was approximately $2.2 \mu\text{Ci} \cdot \mu\text{g protein}^{-1}$.

2.18 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS/PAGE)

Slab gel electrophoresis was performed according to the method of Laemmli (1970) with the modification of Douglas and Butow (1976) and other modifications as stated.

2.18.1 Solutions for SDS/PAGE

A. Separating gel buffer: 1.5 M Tris-HCl buffer, 8 mM EDTA, 0.4% (w/v) SDS, pH 8.8.

B. Stock acrylamide: 30% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene bisacrylamide, dissolved in water, filtered through glass wool and stored at 4°C in the dark.

C. Stacking gel buffer: 0.5 M Tris-HCl buffer, 8 mM EDTA, 0.4% (w/v) SDS, pH 6.8.

D. Polyacrylamide: 1.5% (w/v) polyacrylamide dissolved slowly in water and containing 1 mM sodium fluoride and 1 mM sodium azide.

E. Concentrated sample buffer: 0.2 M Tris-HCl buffer, 8 mM EDTA, 8% (w/v) SDS, 40% (v/v) glycerol, 4% 2-mercaptoethanol, 0.0025% (w/v) bromophenol blue, pH 6.8. This was four times concentrated and was added to samples in solution or used to dissolve solid samples but was always diluted 4 fold in the final sample.

F. Electrode buffer: 50 mM Tris-glycine buffer, 2 mM EDTA, 0.1% (w/v) SDS, pH 8.6.

2.18.2 Preparation of gel cassette and spacer gel

The glass plates (18.5 x 20 cm) were washed thoroughly, rinsed with distilled water and wiped with tissue soaked in acetone. The plastic spacers (approximately 1 mm thick), cleaned with acetone, had a thin coat of high vacuum silicone grease applied down one edge on both sides. They were applied to the edges of one plate with grease-coated edge to the outside and the cassette completed by the application of the second plate on top.

To seal the bottom of the cassette, a trough made with aluminium foil was placed around the bottom of the cassette and 12 ml of a 20% (w/v) acrylamide solution containing 0.2% (w/v) SDS and 0.4 M Tris-HCl buffer pH 8.8, with 10 μ l TEMED and 0.23 ml 10% (w/v) ammonium persulphate added, was poured in. After polymerisation, the trough and excess gel were removed from the bottom of the cassette.

2.18.3 Preparation of separating gels

For 30 ml of 10% acrylamide gel, 10 ml solution B was mixed with 7.5 ml solution A, 5 ml solution D and 7.2 ml distilled water. For other concentrations of acrylamide the amount of solution B was varied and

the final volume made up to 30 ml with distilled water. Polymerisation was initiated by the addition of 15 μ l TEMED and 0.3 ml 10% (w/v) ammonium persulphate. The solution was quickly poured into the gel cassette, leaving space for the stacking gel, overlaid with water-saturated secondary butanol, and left for 1 h to set.

2.18.4 Preparation of exponential gradient separating gel (6-12%)

A small mixing chamber was filled with 15 ml 12% acrylamide gel solution, made by the procedure described in Section 2.18.3, and 7.5 μ l TEMED and 80 μ l 10% (w/v) ammonium persulphate were added. Into this was pumped 15 ml 6% acrylamide gel solution containing 7.5 μ l TEMED and 80 μ l 10% (w/v) ammonium persulphate at the same rate as the diluted mix was delivered to the cassette (twin-channel pump). The total pouring time was 20 min and the gel was overlaid with water-saturated secondary butanol and left for 1 h to set.

2.18.5 Preparation of stacking gel

The stacking gel was 4.5% acrylamide and was prepared by mixing 1.2 ml solution B, 2.0 ml solution C, 1.3 ml solution D, 3.4 ml distilled water, 5 μ l TEMED and 75 μ l 10% (w/v) ammonium persulphate. The water-saturated secondary butanol was washed off the separating gel, the stacking gel poured in and the spacer comb inserted. After 30 min the spacer comb was removed and the cassette was clamped in the electrophoresis tank and used that day.

2.18.6 Sample preparation for SDS/PAGE

Samples containing 50-100 μ g protein had an equal volume of 20% (w/v) TCA added to them, were vortexed and left for 10 min before centrifugation at 1000 x g for 10 min. The pellets were washed with 10% (w/v) TCA and then acetone before drying by vacuum desiccation for 3-4 h. The pellets were dissolved in 50 μ l diluted solution E and heated in a boiling water bath for 5 min. Some pellets from very hydrophobic proteins were

dissolved in 45 μ l of a solution of 10% (w/v) SDS and 8 M urea, with 15 μ l solution E added, followed by boiling as above. Standards used were β -galactosidase (M_r = 125 000), phosphorylase a (92 000), bovine serum albumin (66 000), γ -globulin (50 000 and 23 500), ovalbumin (43 000) and cytochrome c (11 700).

2.18.7 Fixing, staining, destaining and drying of gels

Gels were fixed in 10% (v/v) glacial acetic acid, 10% (v/v) methanol for 30 min at 37°C, followed by staining for 30 min at 37°C in 7.5% (v/v) glacial acetic acid, 50% (v/v) methanol containing 0.25% (w/v) PAGE Blue 83 (Sigma). Gels were then destained with 7% (v/v) glacial acetic acid, 10% (v/v) methanol for about 2 h at 55°C, with some pure white wool included to accelerate the process.

The destained gels were applied to wet 3 MM Whatman chromatography paper and dried on a gel drier (Hoefer Scientific Instruments).

2.19 32 P-LABELLING OF PROTEIN

Samples containing about 100-200 μ g protein in 10 mM Tris-HCl buffer pH 7.0 at 37°C containing 0.5 mM DTT were incubated with a labelling mixture containing 1.3 mM $[\gamma\text{-}^{32}\text{P}]$ ATP, 10 mM magnesium acetate, 100 μ M cAMP and 50 mM Tris-HCl buffer pH 7.4 at 37°C for 20 min at 37°C.

In some cases exogenous cAMP-dependent protein kinase (\sim 10 μ g, type II bovine heart) in 20 mM Tris-HCl buffer pH 7.4 at 37°C containing 1 mM DTT, or catalytic subunit of cAMP-dependent protein kinase (\sim 100-150 units) in a 0.3 M DTT solution were also included in the incubations.

The reactions were terminated by the addition of an equal volume of ice-cold 25% (w/v) TCA and the samples prepared for SDS/PAGE as described in Section 2.18.6.

2.20 $[1,3\text{-}^3\text{H}]$ DFP-LABELLING OF PROTEIN

Samples containing 100-200 μ g protein in 10 mM potassium phosphate buffer pH 7.4 containing 0.2 mM DTT had $[1,3\text{-}^3\text{H}]$ DFP in propylene glycol

added to them to a final concentration of about 100 μM . The incubation was for 30 min at 37°C and the reaction terminated by the addition of an equal volume of ice-cold 25% (w/v) TCA and the samples prepared for SDS/PAGE as described in Section 2.18.6.

2.21 DETERMINATION OF RADIOACTIVITY IN GELS BY AUTORADIOGRAPHY

For ^{32}P -labelling experiments, the dried gels were exposed to Agfa-Gevaert Curix RP1 X-ray film for periods of 24 h to 1 week depending upon intensity of labelling. In some cases the process was accelerated by presensitising the film (Rogers (1979)) with a single flash from a Braun Hobby 17B flashgun through a Wratten No. 22 filter at a distance of 1 metre, followed by exposure to the gel between calcium tungstate sheets (Dupont Cronex lighting plus intensifying screens, Dupont (U.K.), Stevenage, Herts.) at -70°C (Laskey and Mills (1977), Swanstrom and Shank (1978), Rogers (1979)).

For ^3H -labelling experiments, the radioactivity was detected by fluorography and autoradiography (Chamberlain (1979)). The gels were fixed, stained and destained as described in Section 2.18.7 and then soaked in 10 volumes distilled water for 1 h. The gels were then soaked in 10 volumes 1.0 M sodium salicylate for 1 h and dried as described in Section 2.18.7. The gels were exposed to presensitised X-ray film with intensifying screens at -70°C (as above) for 1 month.

The films were developed for 5 min with Agfa-Gevaert G150 developer, soaked in 5% (v/v) acetic acid stopper for 5 min and fixed with Agfa-Gevaert G33A/Aditan for 5 min.

2.22 DETERMINATION OF RADIOACTIVITY IN GEL SLICES

For ^{32}P -labelling experiments, the appropriate tracks on the dried gel were cut out and divided into 1 mm slices using a sharp scalpel. The slices were placed on the bottom of scintillation vials (2.5 x 5.5 cm), covered with 500 μl distilled water and left to soak for 4 h.

The vials had 10 ml Triton X-100/toluene scintillation cocktail added to them and the radioactivity determined in a Packard Tri-Carb liquid scintillation spectrometer.

For ^3H -labelling experiments, the radioactivity was estimated essentially as described by Basch (1968).

The gels were fixed, stained and destained as described in Section 2.18.7. The appropriate tracks were cut from the wet gel and sliced into 1 mm sections by a Mickle gel slicer. The slices were placed on the bottom of scintillation vials (2.5 x 5.5 cm) and covered with 500 μl of a mixture of 90% NCS tissue solubiliser/10% water (v/v). The vials were capped and incubation continued for 3 h at 65°C . After cooling, 10 μl concentrated HCl was added to the vials to neutralise the strongly basic NCS solution followed by 15 ml toluene scintillation cocktail (toluene containing 4 g l^{-1} PPO and 0.03 g l^{-1} POPOP). The vials were stored for 7-10 days in the dark to allow chemiluminescence to subside (Peng (1977)) and the radioactivity estimated in a Packard Tri-Carb liquid scintillation spectrometer.

2.23 PURIFICATION AND RECRYSTALLISATION OF CHOLATE

Cholic acid (2.5 g) was added to 1 litre 30% (v/v) ethanol and the mixture heated to dissolve the cholic acid. Activated charcoal (0.5 g) was added and the mixture refluxed for 10 min. The mixture was removed from heat and celite (1 g) added. About 100 ml 30% (v/v) ethanol containing celite (100 mg) was heated, poured onto a large glass sinter and the hot cholate mixture poured over. The filtered cholate solution was cooled overnight at 4°C . The cholate crystals were filtered (Whatman No. 1) and dried by vacuum desiccation. The yield was calculated to be 80%.

Purified cholic acid (5 g) was heated with 10 ml distilled water and 5 M NaOH added dropwise to convert the acid to the sodium salt.

The resulting, clear solution of sodium cholate ($\sim 500 \text{ mgml}^{-1}$) had a pH of 7.6, and was used as a stock for preparing the buffers described later.

2.24 SERUM CORTICOSTERONE MEASUREMENTS

Serum corticosterone was measured by radioimmunoassay (Ruder, Guy and Lipsett (1972)).

2.25 MEASUREMENT OF THE CHOLESTEROL CONTENT OF BOVINE ADRENOCORTICAL LIPID DROPLETS

Total cholesterol was determined by the cholesterol oxidase method (Richmond (1973)).

2.26 PROTEIN DETERMINATION

Protein concentrations were estimated by the method of Bradford (1976), or in some early experiments, by the method of Lowry, Rosebrough et al. (1951). Crystalline bovine serum albumin was the protein standard in every determination.

2.27 STATISTICAL ANALYSIS

Where indicated, the statistical significance of the difference was tested using the Student t test. Unless otherwise indicated, the difference between groups was significant at $p < 0.05$.

SECTION 3PURIFICATION OF CHOLESTEROL ESTER HYDROLASE FROM BOVINEADRENOCORTICAL CYTOSOL I - DETERGENT SOLUBILISATION

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3.1 INTRODUCTION

Cholesterol ester hydrolase (CEH) from bovine adrenocortical cytosol has been found to be very difficult to purify (Gorban (1980)). One of the main reasons for this is that, although the enzyme is cytosolic, and thus 'soluble', it was found to be associated with lipids and heavily aggregated giving the enzyme an apparently high molecular weight (Beckett (1975), Beckett and Boyd (1977), Naghshineh, Treadwell et al. (1978)). Gel permeation chromatography of the enzyme on Sepharose 4B (Beckett and Boyd (1977), Wallat and Kunau (1976)), 6% agarose gel (Pittman and Steinberg (1977)), Sephadex G-200 (Wallat and Kunau (1976)) and Biogel A150 m (Gorban (1980)) resulted in the activity being divided between peaks in the void volume and within the fractionation range of the column. The relative proportion of the enzyme activity in each peak depended upon the particular gel matrix used. In all cases in which gel permeation chromatography was used the void volume fraction had higher specific activity, however pooling this fraction resulted in poor recoveries. Mild delipidation of the enzyme by cold butanol/acetone did not significantly alter the elution profile on Biogel A150 m or provide any increased purification, but 50% of the enzyme activity was lost after 4 days storage at -18°C (Gorban (1980)). The relationship between the two peaks has not been fully investigated but they probably represent aggregates of different size where the lipid:protein ratio is different. Pittman, Golanty and Steinberg (1972) reported that hormone-sensitive lipase (HSL) from rat adipose tissue was eluted from a 4% Biogel column in two peaks with the excluded peak having a density ($d < 1.2$) that was less than the retarded peak. Later Pittman and Steinberg (1977) found that CEH and triacylglycerol lipase in rat adrenal cytosol were eluted from a 6% Biogel column in two peaks and the density of the retained fraction was greater than that of the excluded peak.



Gorban (1980) showed that CEH and triacylglycerol lipase activities from bovine adrenocortical cytosol were eluted, in parallel, from Biogel A150 m in two peaks, and the excluded peak was found to float at a density of $d\ 1.2$ in a sucrose gradient. From all these studies it was concluded that CEH in bovine adrenocortical cytosol was present in protein:lipid aggregates with a wide range of molecular weights and differing densities. The poor recoveries also seriously hampered purification by gel permeation and flotation techniques.

Detergents have been used for many years to solubilise membrane proteins (reviewed Helenius and Simons (1975), Tanford and Reynolds (1976)) but the use of detergents with cytosolic proteins was rare. However a monoacylglycerol lipase activity from rat adipose tissue cytosol had been purified 2 500-fold by a method including detergent solubilisation (Tornqvist and Belfrage (1976)). In this case a pH 5.2 precipitate prepared from the cytosol was sonicated with the non-ionic detergent Nonipol TD 12. This solubilisation procedure was then used with some success in the purification of HSL from rat adipose tissue cytosol (Belfrage, Jergil et al. (1977)). Solubilisation of HSL by Triton X-100 has also been used in the purification of the enzyme from chicken adipose tissue cytosol (Berglund, Khoo and Steinberg (1979)). However detergent solubilisation had not been attempted in the purification of CEH from adrenocortical cytosol. The aim of this section of work was to investigate the effect of detergents upon CEH activity from bovine adrenocortical cytosol, and to develop a purification procedure involving detergent solubilisation.

The enzyme preparation used in this work was an ammonium sulphate (0-40%) precipitate prepared from the cytosol. Ammonium sulphate precipitation had been shown to be an effective early, batch-wise step in the purification of the enzyme from bovine adrenal cortex

(Trzeciak and Boyd (1979), Beckett and Boyd (1977), Naghshineh, Treadwell et al. (1978)) giving up to 4-fold purification with at least 50% recovery. Many researchers studying HSL in adipose tissue use a pH 5.2 precipitation as the early batch-wise step (Huttunen, Steinberg and Mayer (1970), Belfrage, Jergil et al. (1977)). However Wallat and Kunau (1976) compared ammonium sulphate precipitation with acid precipitation in the purification of CEH from bovine adrenocortical cytosol and the results showed that ammonium sulphate precipitation was more effective in both purification and recovery. Therefore the ammonium sulphate precipitation method was used in all purifications investigated in this study. This method consistently gave purifications of about 4-fold with recoveries of 65% or higher (see Tables 3.1 and 5.1).

3.2 PRELIMINARY INVESTIGATION OF A RANGE OF DETERGENTS

One method to investigate the effectiveness of a detergent at solubilising CEH would be the elution profile of the enzyme activity after gel permeation chromatography before and after treatment with detergent. This would be time consuming as each column would have to be equilibrated in buffer containing the appropriate detergent. Furthermore this method does not account for the many problems associated with the use of detergents. Firstly, the detergent may inhibit the enzyme activity. Secondly, the detergent may disrupt the cholesterol oleate substrate emulsion and make assay of the enzyme difficult. Lastly the detergent may solubilise the unhydrolysed cholesterol $[1-^{14}\text{C}]$ oleate during the extraction phase of the enzyme assay and hence interfere with the assay. For these reasons it was decided to use a quick and simple test for the suitability of the detergents.

A range of detergents including anionic, cationic and various types of non-ionic detergents were investigated to determine their

effect on the enzyme. The enzyme preparation used was the ammonium sulphate (0-40%) precipitate prepared from bovine adrenocortical cytosol. The cytosol had 80% saturated ammonium sulphate solution in 50 mM potassium phosphate buffer pH 7.4 added slowly and with stirring, at 4°C, to a final ammonium sulphate saturation of 40%. The precipitate was collected by centrifugation at 6000 x g for 20 min, resuspended in 20 mM potassium phosphate buffer pH 7.4 containing 0.2 mM EDTA and 1 mM DTT and dialysed overnight against 250 volumes of this buffer. Samples of this preparation were mixed with detergent at a range of detergent:protein (mg:mg) ratios from 0-2 which is the range in which most solubilisations are effective (Helenius and Simons (1975)). The samples were sonicated by a Rapidis 150, power rating 3 for 80s (4 x 20s) at 4°C. Aliquots were taken and assayed for CEH activity, and the results are shown in Fig. 3.1.

A wide range of detergent effects were observed. Detergents like Tween 40 inhibited the enzyme even at the lowest detergent:enzyme ratios and other detergents such as Triton X-100 and Nonidet P42 inhibited the enzyme at higher ratios of detergent:enzyme. Finally detergents such as sodium cholate and nonyl glucoside increased the enzyme activity over a wide range of detergent:enzyme ratios. The increased enzyme activity could be due to solubilisation of the enzyme resulting in an increase in effective enzyme concentration. However it could also be due to the detergent assisting in the presentation of the substrate to the enzyme by affecting the interactions between the enzyme particles and the emulsion particles. It was not known why some detergents were inhibitory, they may be adversely affecting the protein-substrate interactions or altering the conformation of the enzyme.

Sodium cholate (subsequently denoted simply as cholate) was chosen as the most suitable detergent for further studies as it consistently

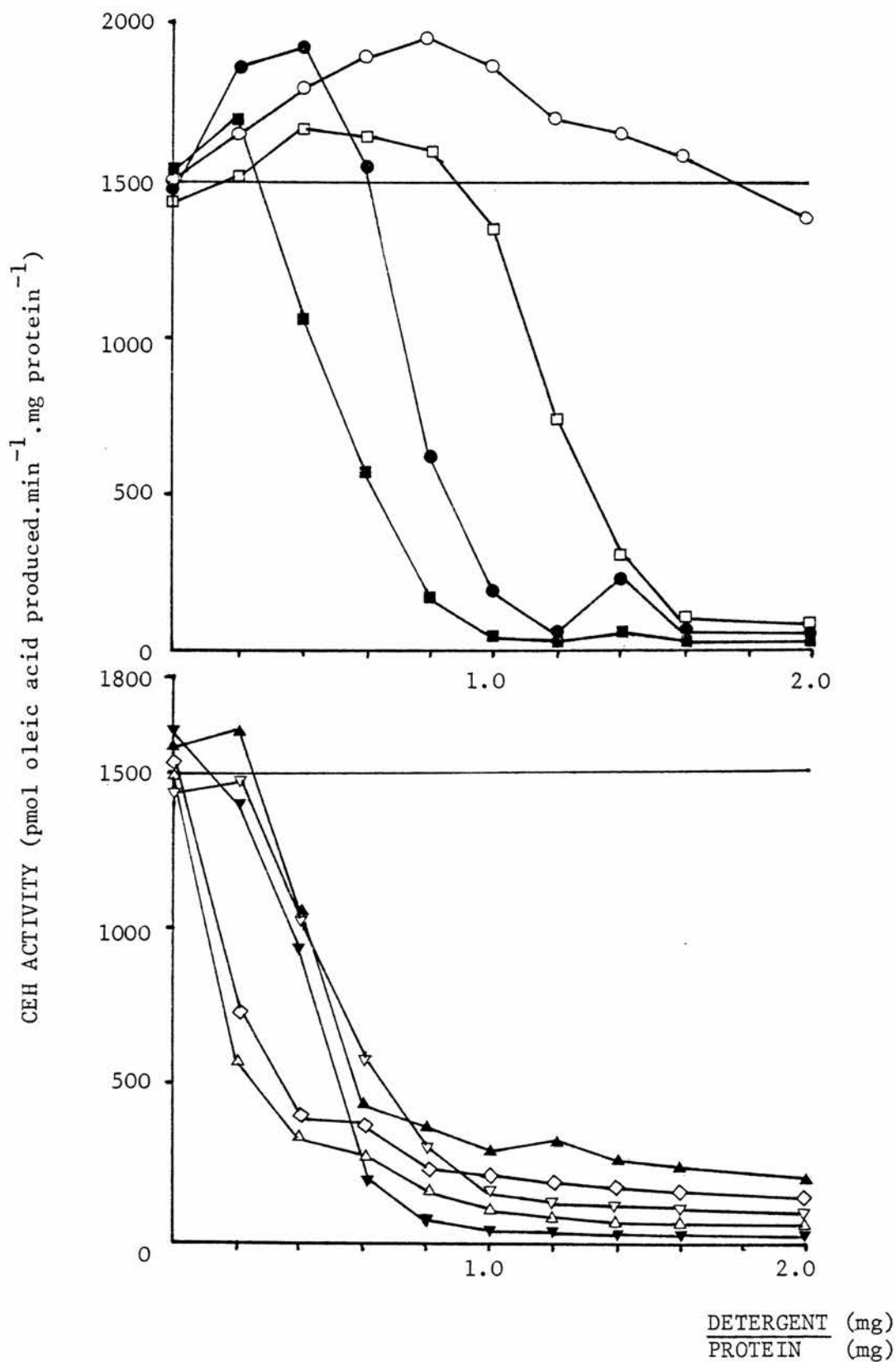


Fig. 3.1.

Legend to Fig. 3.1. Effect of various detergents on the activity of CEH. 1 ml samples of an ammonium sulphate (0-40%) precipitate (5.1 mg ml^{-1} in 20 mM potassium phosphate buffer pH 7.4 containing 0.2 mM EDTA and 1 mM DTT) prepared from bovine adrenocortical cytosol had various detergents (20 mg ml^{-1} in the above buffer) added to them to the final mg detergent:mg protein ratios shown. The samples were sonicated as described in the text and then assayed for CEH activity as described in the methods section.

○, Cholate; ●, Nonidet P42; □, Nonyl-glucoside; ■, Triton X-100; ▲, Deoxycholate; △, Tween 40; ▽, Lubrol PX; ▼, Brij 36T; ◇, Saponin.

gave increased CEH activity over the widest range of detergent:enzyme ratios. Also, as an ionic detergent it is more easily removed than a non-ionic detergent such as nonyl glucoside. Overnight dialysis against 100-fold buffer containing ion-exchange beads (Amberlite XAD-2) was found to be effective at removing cholate (Furth (1980)), whereas non-ionic detergents are notoriously difficult to remove (Helenius and Simons (1975), Tanford and Reynolds (1976)). The cholate used in the following experiments was purified and recrystallised as described in the methods section.

3.3 FURTHER INVESTIGATION OF CHOLATE SOLUBILISATION

3.3.1 Effect of sonication

If sonication of the enzyme with cholate resulted in breaking up the enzyme:lipid aggregates, then varying the time of sonication may have an effect on the degree of solubilisation. A sample of an ammonium sulphate (0-40%) precipitate from bovine adrenocortical cytosol prepared in 20 mM potassium phosphate buffer pH 7.4 containing 0.2 mM EDTA and 1 mM DTT had cholate added to 0.8 mg cholate:mg protein. This was found to be the most effective solubilisation ratio (Fig. 3.1). The mixture was sonicated, as before, and samples removed at various times and assayed for CEH.

Fig. 3.2 shows that enzyme activity increased with increasing sonication up to 60 s, but after this point no change in activity was seen with further sonication. It was possible that the initial increase in activity over the first 60 s sonication was due to increasing solubilisation.

3.3.2 Stability of the enzyme in buffers containing cholate

Although CEH was not inhibited by cholate treatment in these short term experiments, any purification procedure would require the enzyme to be maintained in buffers containing cholate for much longer time

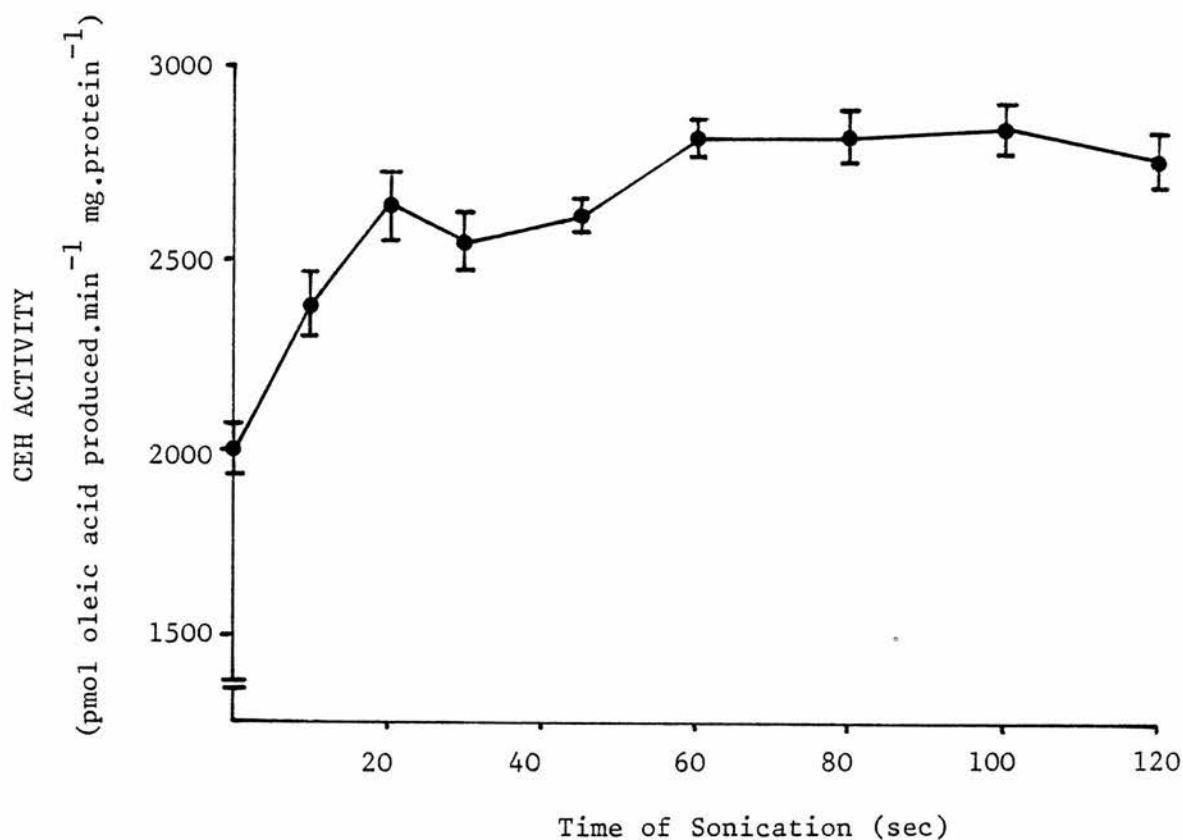


Fig. 3.2. Effect of time of sonication with cholate on CEH activity. An ammonium sulphate (0-40%) precipitate prepared from bovine adrenocortical cytosol in 20 mM potassium phosphate buffer pH 7.4 containing 0.2 mM EDTA and 1 mM DTT had cholate (500 mgml⁻¹ in distilled water) added to it to a final ratio of 0.8 mg cholate:mg protein. The mixture was sonicated for 120s in 10s intervals. After each interval triplicate samples were assayed for CEH activity. Each point is the mean \pm S.E.M. from two experiments.

periods. To investigate the stability of the enzyme under such conditions, a sample of an ammonium sulphate (0-40%) preparation had cholate added to 0.8 mg cholate:mg protein and was sonicated for 80 s as described in Section 3.2. Samples were withdrawn and the CEH activity measured at various times up to 72 h after sonication and storage at 4°C or -20°C (see Fig. 3.3). Parallel incubations containing no cholate and cholate plus 10% (v/v) and 20% (v/v) glycerol in the buffers were also performed.

Fig. 3.3 shows that short term exposures to cholate of up to 6 h had relatively little effect on the enzyme activity but by 24 h the activity was reduced by 35%. The loss was about 70% and 90% after 48 h and 72 h respectively. In the control sample without cholate only 10% of the CEH activity was lost over the 72 h period. However the inclusion of glycerol in the buffer protected the enzyme against the inhibitory effects of cholate. In the experiment shown no loss of enzyme activity was observed when glycerol was included. In fact, after 72 h the enzyme activity was slightly higher and on repeating the experiment the loss of activity due to cholate in the presence of glycerol was never more than about 10% compared to the control without cholate. There was very little difference between 20% (v/v) and 10% (v/v) glycerol although 20% glycerol was slightly more effective in the long term. In all subsequent work with cholate, glycerol (10% or 20%) was included in all the buffers used.

3.3.3 Effect of cholate upon the activation and deactivation of CEH

One main reason for purifying CEH was to study the modulation of its activity in vitro using purified components and therefore it was essential that the purification of the enzyme did not result in a preparation that could not be phosphorylated. This may occur if the native enzyme was present as subunits with different subunits responsible

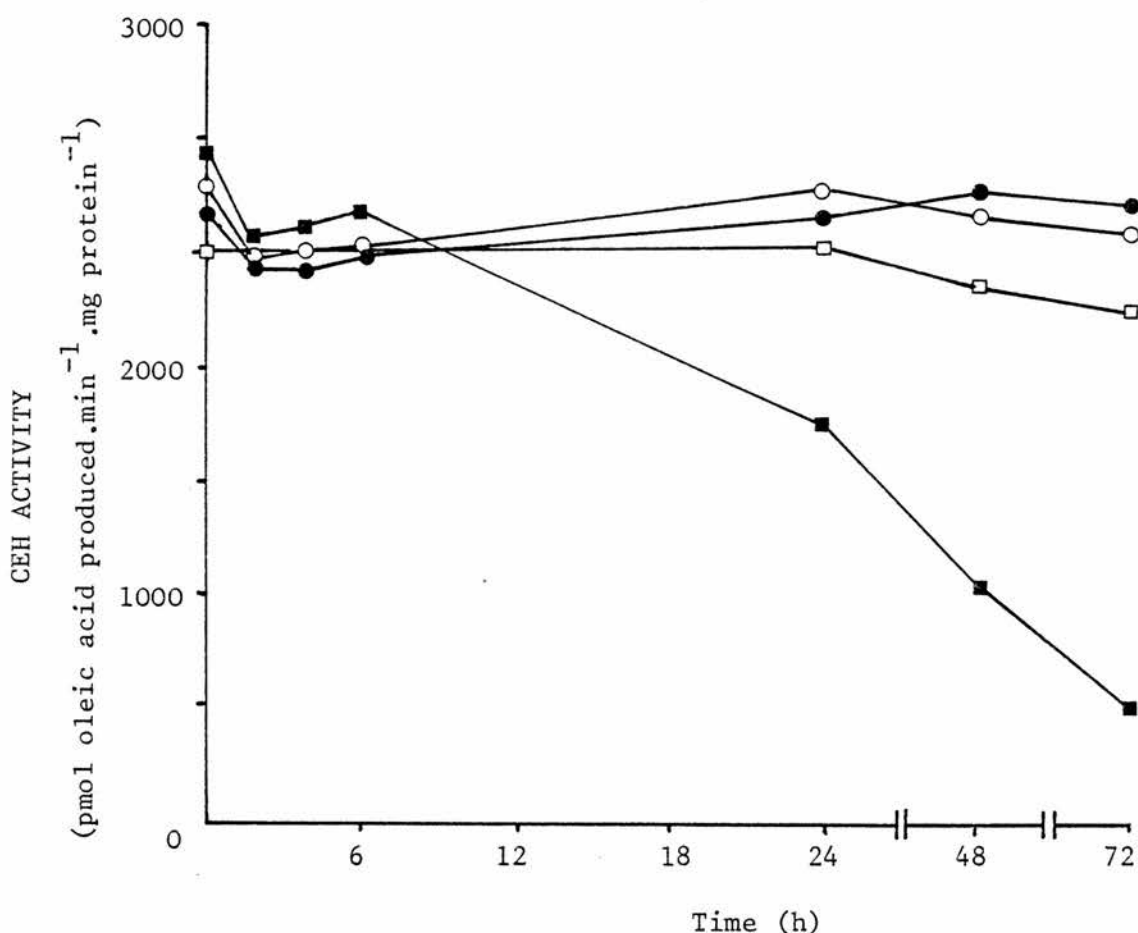


Fig. 3.3. Stability of CEH in buffers containing cholate.

An ammonium sulphate (0-40%) precipitate prepared from bovine adrenocortical cytosol in 20 mM potassium phosphate buffer pH 7.4 was sonicated for 80s at 4°C with cholate (20 mgml⁻¹ in above buffer) at a ratio of 0.8 mg cholate:mg protein. Samples were assayed for CEH activity at various time intervals. Parallel experiments, in which the buffers also contained 10% (v/v) or 20% (v/v) glycerol and a control without cholate were also carried out. The incubations were maintained at 4°C during the sampling period and at -20°C between the longer time periods.

□, Control; ■, Cholate; ○, Cholate plus 10% (v/v) glycerol; ●, Cholate plus 20% (v/v) glycerol.

for catalytic activity and regulation of activity. To investigate the effect of cholate treatment on the modulation of the enzyme activity a sample of an ammonium sulphate (0-40%) precipitate from bovine adrenocortical cytosol was sonicated with cholate at a ratio of 0.8 mg cholate:mg protein for 80 s at 4°C. Samples were taken immediately and also after 48 h and the CEH activity measured after preincubation in basal, deactivation and activation buffers as described in the methods section.

Fig. 3.4 shows that the presence of cholate did not affect the deactivation of the enzyme compared to the control sample even after 48 h. Conversely the activation of the enzyme was reduced in the presence of cholate. However these results suggest that cholate does not dissociate the catalytic activity from the phosphorylation site as incubation with Mg^{2+} significantly lowered the specific activity. The effect of cholate on the activation process might reflect the effect of the detergent upon the activity of cAMP-dependent protein kinase but this aspect of the problem was not studied further.

3.3.4 Sepharose CL-4B chromatography

The experiments so far had indicated that cholate solubilised CEH and that it could be useful in future purifications. To obtain direct evidence that the enzyme activity was solubilised by cholate treatment it was necessary to investigate the elution profile of the enzyme when subjected to gel permeation chromatography on an appropriate gel medium. An ammonium sulphate (0-40%) precipitate from bovine adrenocortical cytosol prepared in 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA, 0.1 mM DTT and 10% (v/v) glycerol (buffer A) had cholate added to it to a ratio of 0.8 mg cholate:mg protein. The solution was mixed and sonicated on a Rapidis 150, power rating 7 for 40 s (4 x 10 s) at 4°C. The sonicated enzyme was applied to a Sepharose CL-4B

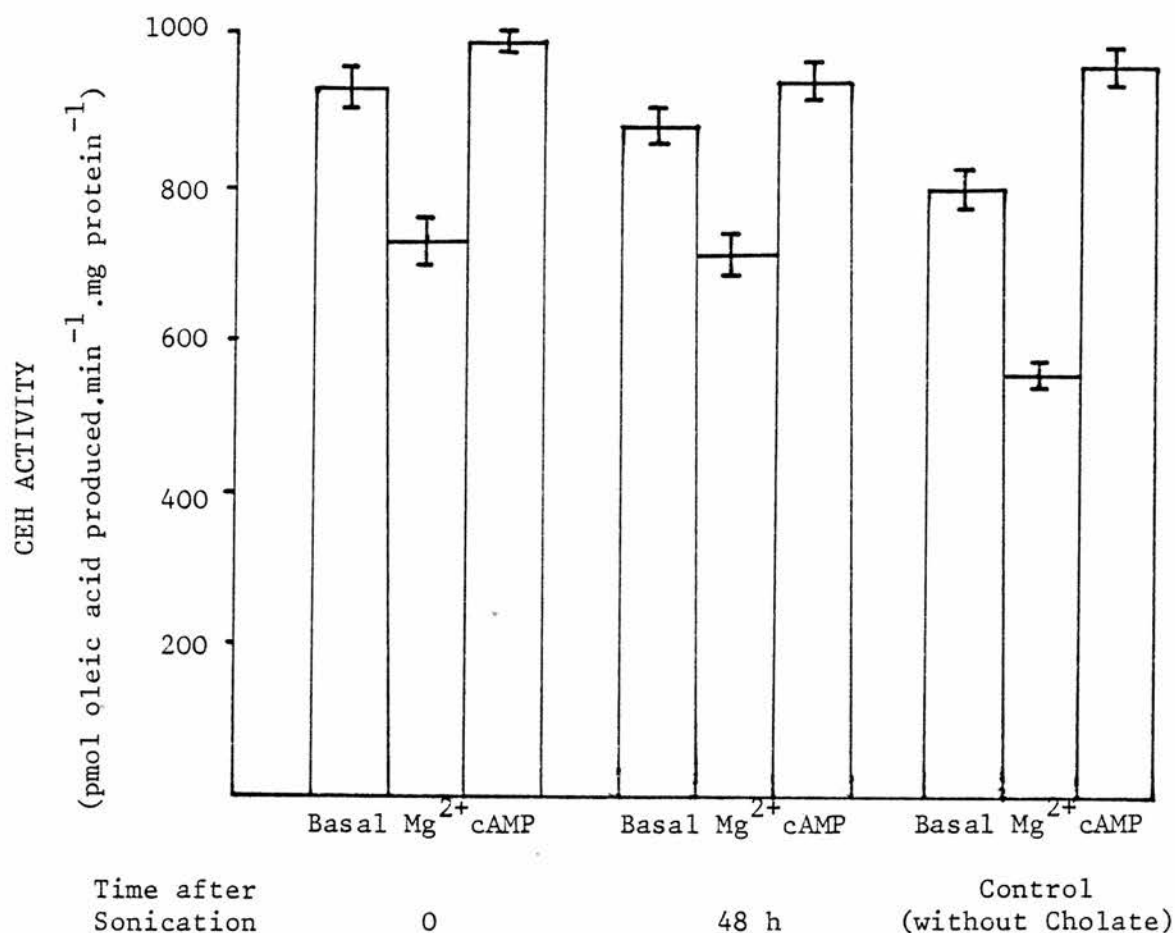


Fig. 3.4. Effect of cholate upon activation and deactivation of CEH. An ammonium sulphate (0-40%) precipitate prepared from bovine adrenocortical cytosol in 20 mM potassium phosphate buffer pH 7.4 containing 0.2 mM EDTA, 1 mM DTT and 10% (v/v) glycerol was sonicated for 80s at 4°C with cholate (20 mgml⁻¹ in above buffer) at a ratio of 0.8 mg cholate:mg protein. CEH activity was assayed immediately after sonication and after 48 h, following preincubation of the solution with basal, deactivation (Mg²⁺) or activation (cAMP) buffers as described in the methods section. A control sample without cholate was assayed in the same way.

column at 4°C, equilibrated in 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol and 0.1% (w/v) cholate (buffer B) and eluted with this buffer.

Fig. 3.5a shows that in the absence of cholate the CEH activity was eluted partly in the void volume and partly in a broad peak spread throughout the fractionation range of the column. After cholate treatment and gel filtration in the presence of cholate virtually all the CEH activity eluted from the column in a peak at about $1.8 \times V_o$ (Fig. 3.5b). The protein elution profile was also altered with the void volume peak observed in the absence of detergent, greatly reduced leaving, in the presence of detergent, a single, large broad peak at about $2.0 \times V_o$. The CEH activity eluted before the peak fractions of protein and showed a 2-3 fold increase in specific activity compared to the ammonium sulphate precipitate before the addition of cholate (Table 3.1). The fractions indicated were pooled and termed "cholate solubilised enzyme". This was used in the following purification procedure.

3.4 PURIFICATION OF CEH FROM BOVINE ADRENOCORTICAL CYTOSOL USING CHOLATE SOLUBILISATION AND CONVENTIONAL CHROMATOGRAPHIC TECHNIQUES

The results obtained with Sepharose CL-4B chromatography indicated that cholate solubilised the CEH activity and suggested that a purification procedure using conventional techniques with buffers containing cholate could be successful. A related enzyme, HSL from chicken adipose tissue had been substantially purified using techniques similar to this (Berglund, Khoo and Steinberg (1979)). In the following procedure the cholate solubilised enzyme was chromatographed on hydroxylapatite and DEAE-cellulose and the overall purification is summarised in Table 3.1. Steps I-IV were performed as described in Section 2 (I), Section 3.2 (II) and Section 3.3.4 (III and IV) and all steps were carried out at 4°C.

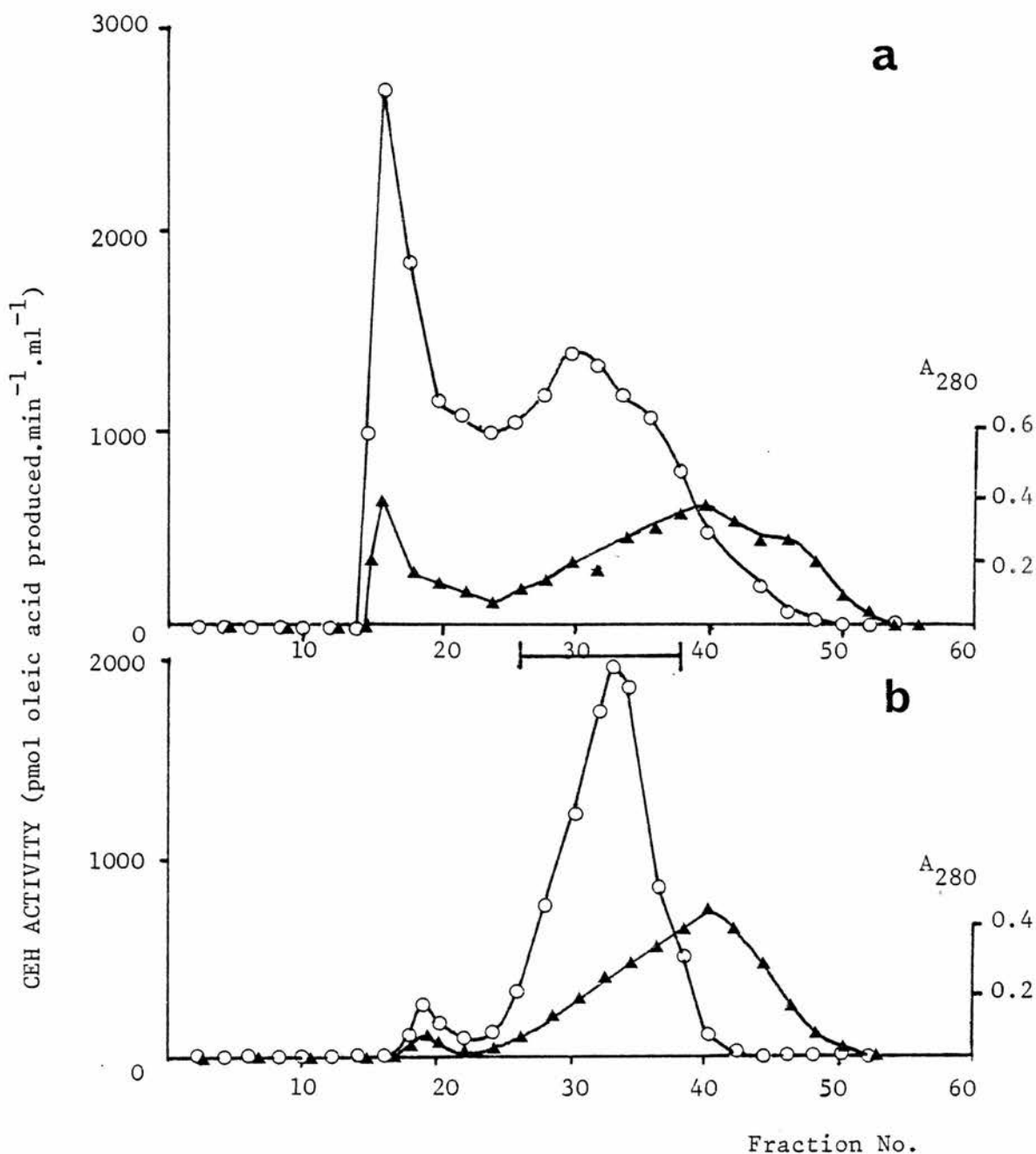


Fig. 3.5. Elution of CEH activity on Sepharose CL-4B before and after cholate treatment.

a) An ammonium sulphate (0-40%) precipitate (17 mg) prepared from bovine adrenocortical cytosol in 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA, 0.1 mM DTT and 10% (v/v) glycerol was applied to a Sepharose CL-4B column (2 x 96 cm) equilibrated and eluted with this buffer.

b) An ammonium sulphate (0-40%) precipitate (10 mg) prepared as above had 0.8 mg cholate per mg protein added, and was sonicated as described in the text. The sample was applied to a Sepharose CL-4B column (2 x 96 cm) equilibrated and eluted with the above buffer containing 0.1% (w/v) cholate.

○, CEH activity; ▲, A₂₈₀; —, Fractions pooled.

Stage	Total Protein (mg)	CEH Activity		Purification (fold)	Recovery (%)
		Specific (units. $\frac{\text{mg}}{\text{protein}}^{-1}$)	Total (units)		
I. 105 000 x g Supernatant	1510	0.72	1081	-	100
II. Ammonium Sulphate (0-40%) Precipitate	234	2.89	676	4.0	63
III. Solubilised Ammonium Sulphate Precipitate	234	N.D.	N.D.	N.D.	N.D.
IV. Sepharose CL-4B	48.6	4.97	241	7.0	22
V. Hydroxylapatite	20.9	3.32	69	-	6
VI. DEAE-Cellulose I	16.3	2.10	34	-	3
VII. DEAE-Cellulose II	10.2	2.02	21	-	2

Table 3.1. Purification of bovine adrenocortical cytosolic CEH using cholate solubilisation. Details of each step are given in the text. Protein concentration was determined by the method of Lowry, Rosebrough et al. (1951). The enzyme activity was measured as described in the methods section. Enzyme activity units were nmol oleic acid produced. min⁻¹.

Step V - Cholate solubilised enzyme was loaded onto a hydroxylapatite column. The column was washed with 2 volumes of buffer B and the enzyme was eluted with a linear gradient of potassium phosphate (0-0.25 M). CEH activity appeared as a broad peak from 0.15 M to 0.2 M potassium phosphate (Fig. 3.6a). The fractions indicated were pooled and dialysed against buffer B to remove potassium phosphate.

Step VI - The dialysed sample was then applied to a DEAE-cellulose column equilibrated in buffer B. The column was washed with 2 bed volumes of the same buffer and the enzyme eluted by a linear gradient of sodium chloride (0-0.5 M). The CEH activity eluted at 0.3 M sodium chloride and the fractions indicated were pooled (Fig. 3.6b). The sample was diluted 1:1 with buffer B to reduce the sodium chloride concentration to 0.15 M.

Step VII - The diluted sample was applied to a second DEAE-cellulose column equilibrated in buffer B containing 0.15 M sodium chloride. The column was washed with 2 bed volumes of the same buffer and the enzyme eluted with a linear gradient of sodium chloride (0.15 M-0.6 M). The CEH activity eluted at 0.33 M sodium chloride and the active fractions were pooled and dialysed against buffer A to remove sodium chloride and cholate (Fig. 3.6c).

The results of the purification procedure showed that cholate solubilisation was only partly successful when used with ion-exchange techniques. The first four steps led to the production of cholate solubilised enzyme where all the activity was present as aggregates of much lower and more uniform molecular weight. At the same time the specific activity of the enzyme was increased 7-fold, with the ammonium sulphate precipitation step consistently giving a 3-4-fold increase and the gel permeation step giving a further doubling of the specific activity. However Table 3.1 also shows that further chromatography on

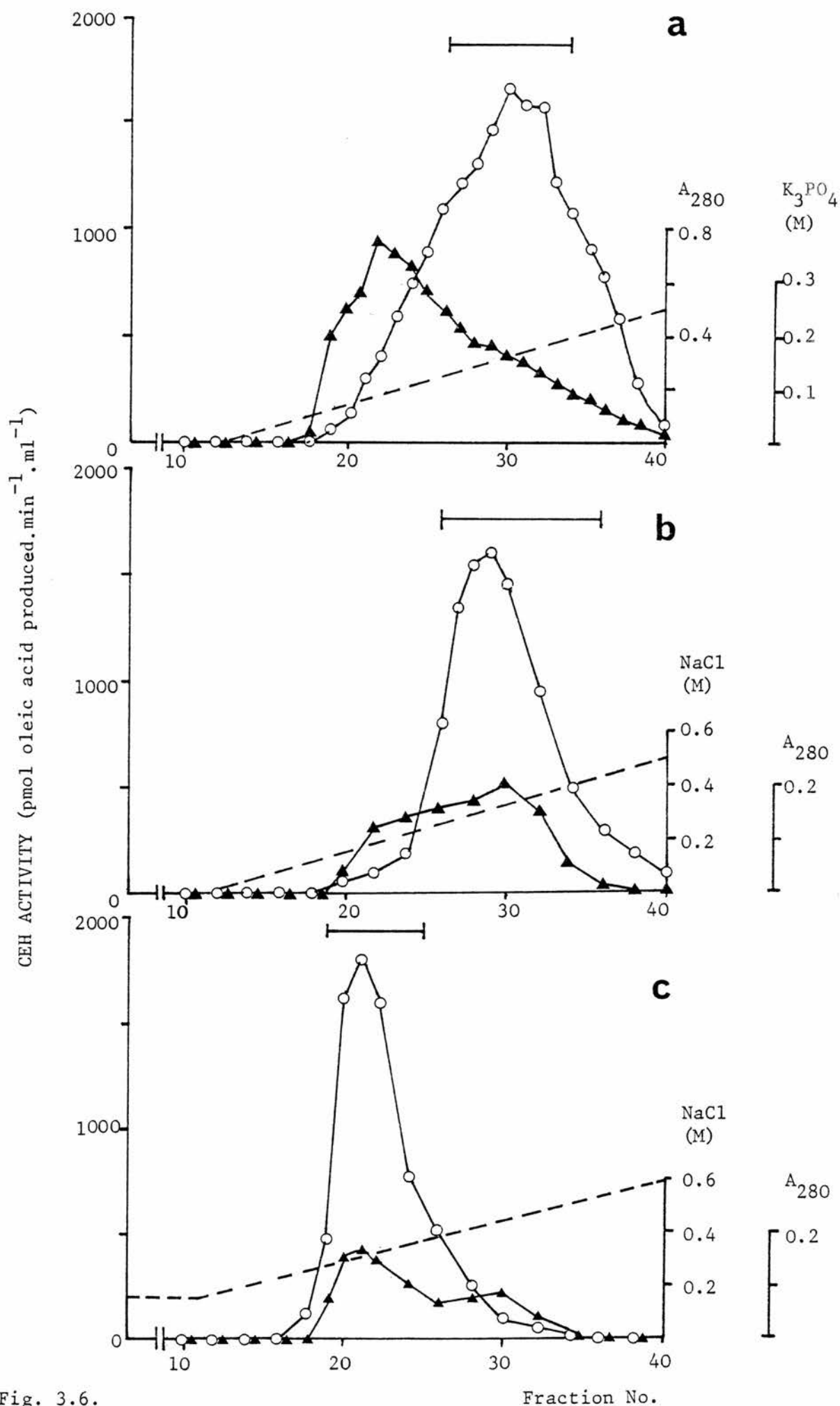


Fig. 3.6.

Legend to Fig. 3.6. Chromatography of cholate solubilised enzyme.

a) Hydroxylapatite chromatography - The column (1.6 x 8 cm) was equilibrated with buffer B and eluted with a linear gradient of potassium phosphate (0-0.25 M) in buffer B in a total volume of 150 ml. The flow rate was 50 ml.h^{-1} and fractions of 5 ml were collected.

b) DEAE-cellulose chromatography I - The column (2.5 x 7.5 cm) was equilibrated with buffer B and the enzyme eluted with a linear gradient of sodium chloride (0-0.5 M) in buffer B in a total volume of 180 ml. The flow rate was 50 ml.h^{-1} and fractions of 6 ml were collected.

c) DEAE-cellulose chromatography II - The column (1.5 x 7.5 cm) was equilibrated with buffer B containing 0.15 M sodium chloride and the enzyme eluted with a linear gradient of sodium chloride (0.15-0.6 M) in buffer B in a total volume of 100 ml. The flow rate was 50 ml.h^{-1} and fractions of 4 ml were collected.

○, CEH activity; ▲, A_{280} ; ---, Salt concentration;

▬, Fractions pooled.

ion-exchange columns resulted in the specific activity of CEH decreasing at each step. This was in contrast to the elution profiles shown in Fig. 3.6 in which the CEH activity eluted in reasonably sharp peaks not fully coinciding with the protein profile. This was clearly the situation on the hydroxylapatite column (Fig. 3.6a), but SDS/PAGE of samples taken from each stage show that only minor purification had been achieved at steps V-VII (Fig. 3.7).

Taken together the results indicate that there were two main reasons for these results. Firstly, the SDS/PAGE of the samples and the elution profiles indicated that cholate, as an ionic detergent, severely interfered in the functioning of the ion-exchange columns used. This resulted in the entire preparation binding to the columns and also eluting virtually en masse, particularly in the DEAE-cellulose steps, with very little difference in the overall protein pattern. Secondly, although the hydroxylapatite step did not appear to be adversely affected by the presence of cholate, the specific activity dropped. This indicated substantial inactivation of the enzyme occurred throughout the later steps. This could be due to prolonged exposure to cholate during both the chromatographic steps and intermediary dialyses, possibly aggravated by the increased detergent:protein ratio in these fractions. This was in contrast to the results obtained in Section 3.3.2, although in that study the enzyme was maintained in a constant environment without the column chromatography effects, increased ionic strength and dialysis that occurred over the 5 day period needed for the purification procedure discussed in this section.

The procedure was repeated, using columns of different dimensions and with QAE-Sephadex A-50 replacing the second DEAE-cellulose step, in an attempt to increase the resolution, but this did not give better results. An attempt was made to minimise the detergent-induced

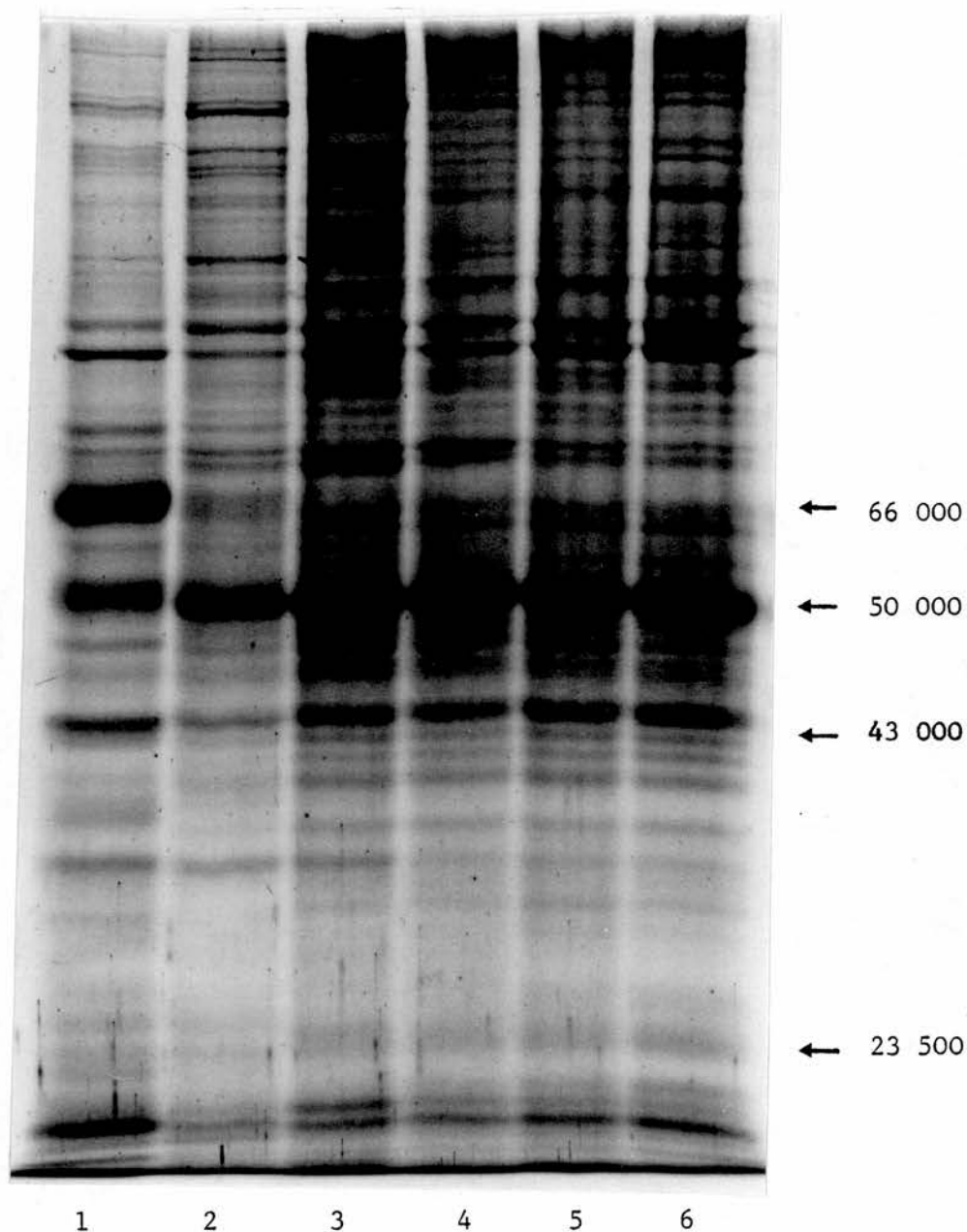


Fig. 3.7. SDS/PAGE of samples from each stage of the purification procedure using cholate solubilised enzyme. Samples containing about 100 μ g protein from each stage were subjected to SDS/PAGE on a 10% gel and stained for protein as described in the methods section. The arrows indicate the position on the gel of molecular weight standards. 1, Tissue cytosol; 2, Ammonium sulphate (0-40%) precipitate; 3, Sepharose CL-4B; 4, Hydroxylapatite; 5, DEAE-cellulose I; 6, DEAE-cellulose II.

inactivation of the enzyme by reducing the cholate concentration in the buffers from 0.1% to 0.02%. This did not affect the elution profiles, and it did not alter the pattern of decreasing specific activity after step IV.

These results indicated that conventional ion-exchange techniques were not compatible with the use of cholate solubilised enzyme in the purification of CEH. Some of the problems associated with using ionic detergents and ion-exchange chromatography had been noted previously (Tanford and Reynolds (1976)). However, despite the problems associated with the use of cholate in lengthy procedures, the cholate solubilisation step was still useful and was used in later experiments (see Section 4).

3.5 THE USE OF HUMAN HDL AND PURIFIED APOLIPOPROTEIN A-I TO SOLUBILISE CEH

Despite the overall failure of the purification procedure described in Section 3.4, the solubilisation of the enzyme remained a crucial step in any purification method. Cholate effectively solubilised the enzyme but there were problems associated with its use.

HSL in the cytosol of adipose tissue was known to be associated with large, lipid-rich complexes, which also contain diacylglycerol, monoacylglycerol and cholesterol ester hydrolase activities (Khoo, Drevon and Steinberg (1980)). These activities cofractionated through limited purification (Berglund, Khoo et al. (1980), Fredrikson, Stralfors et al. (1981a)), and during gel permeation chromatography of crude homogenates on 2% agarose, they all eluted in the void volume (Khoo, Drevon and Steinberg (1980)). However, it had been reported that incubation of crude homogenates from chicken adipose tissue with purified human HDL (hHDL) or apolipoprotein A-I prepared from delipidated hHDL, resulted in dissociation of the large, lipid-rich complexes (Khoo, Drevon and Steinberg (1980)). The HSL activity was now mostly retained and coeluted with ^{125}I -labelled hHDL. The diacylglycerol and

monoacylglycerol hydrolase activities were also largely retained but less so than HSL (74% and 68% retained, respectively). Furthermore, CEH activity was also dissociated from the large aggregates and appeared to elute just prior to HSL. These results suggested that this may be a useful technique to apply to the purification of CEH from bovine adrenocortical cytosol as the enzyme was also present in large, lipid-rich complexes in this tissue (Beckett (1975), Beckett and Boyd (1977)).

HDL was prepared from human serum by sodium phosphotungstate/magnesium chloride precipitation followed by preparative density ultracentrifugation as described in the methods section. To prepare apolipoprotein A-I, a portion of hHDL was delipidated and the crude protein subjected to chromatography on Sephacryl S-200, Sephadex G-100 and Cibacron Blue - Sepharose 4B as described in the methods section.

3.5.1 Effect of hHDL and apolipoprotein A-I on CEH elution profile on gel permeation chromatography

Sepharose CL-4B (4% agarose) had been used in previous purifications of the enzyme and the elution profile of CEH activity using this gel was known (Fig. 3.5a). Therefore this gel was used in the following experiments.

Samples of ammonium sulphate (0-40%) precipitate from bovine adrenocortical cytosol were incubated with hHDL for 4 h at 37°C or with apolipoprotein A-I for 20 h at 4°C and then chromatographed on a Sepharose CL-4B column equilibrated and eluted with 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA and 10% (v/v) glycerol. A control sample of ammonium sulphate precipitate without any preincubation was also chromatographed.

Fig. 3.8a shows that with no preincubation the CEH activity was divided between a large, sharp peak in the void volume and a broad

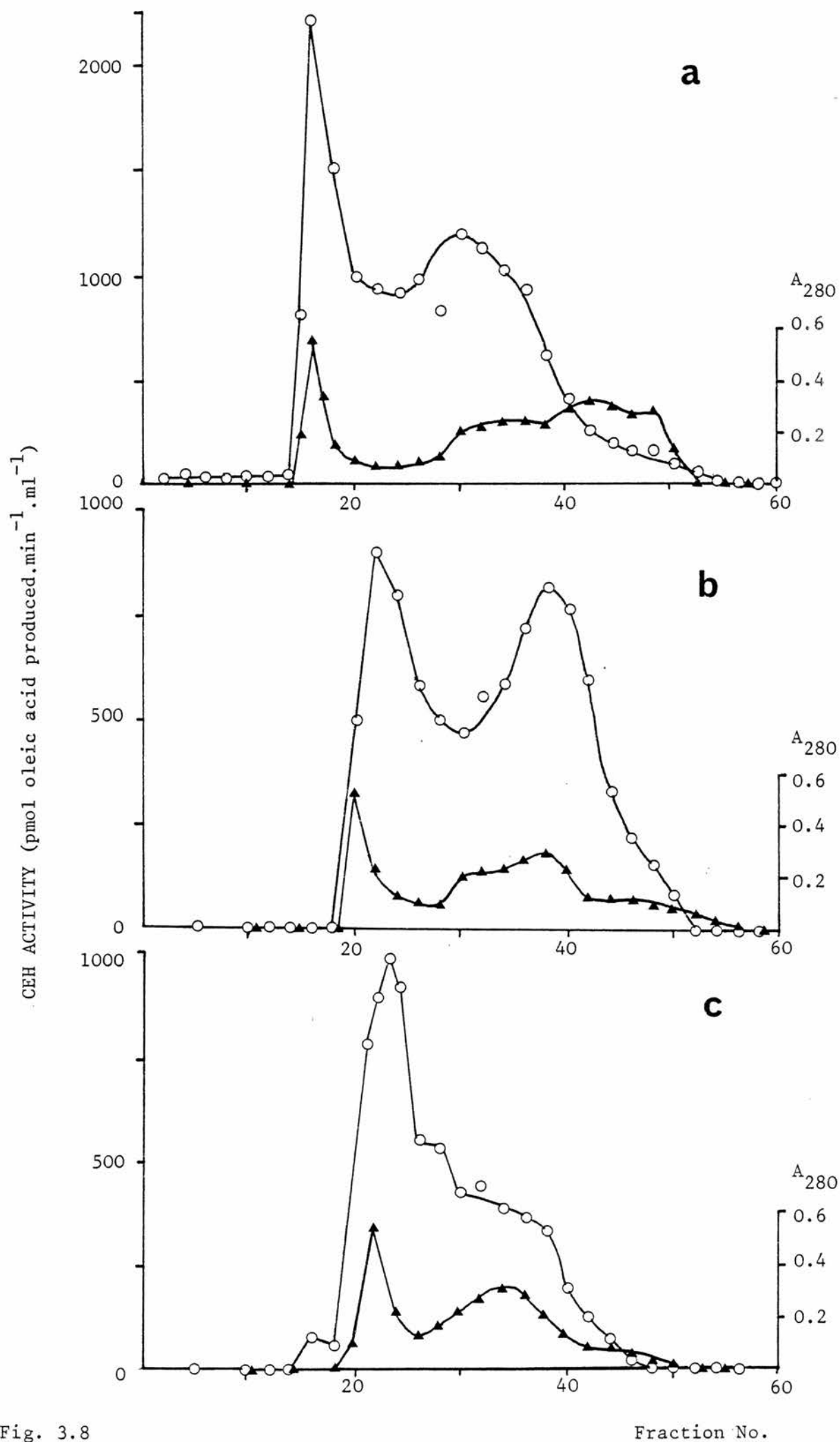


Fig. 3.8

Fraction No.

Legend to Fig. 3.8. Effect of hHDL and apolipoprotein A-I on the elution of CEH from Sepharose CL-4B. The column (1 x 70 cm) was equilibrated and eluted with 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA and 10% (v/v) glycerol (flow rate, 5 ml.h⁻¹; fraction size, 1.5 ml).

a) 1 ml ammonium sulphate (0-40%) precipitate (17 mgml⁻¹) prepared from bovine adrenocortical cytosol in 20 mM Tris-HCl buffer pH 7.4 at 4°C was applied to the column.

b) 1 ml ammonium sulphate precipitate (as above) was incubated with 350 µl hHDL (8 mg protein) for 4 h at 37°C and then applied to the column.

c) 1 ml ammonium sulphate precipitate (as above) was incubated with 2 mg apolipoprotein A-I dissolved in 400 µl of 20 mM Tris-HCl buffer pH 7.4 at 4°C for 20 h at 4°C and then applied to the column.

○, CEH activity; ▲, A₂₈₀.

peak eluting at about $1.8 \times V_0$. Prior incubation with hHDL resulted in an increased amount of CEH eluted in the retarded peak but there was still a substantial amount of activity in the void volume peak, which had been broadened (Fig. 3.8b). Incubation with apolipoprotein A-I caused a reduction in the amount of activity appearing in the retarded peak (Fig. 3.8c). Apolipoprotein A-I, due to its hydrophobic nature, may increase the amount of aggregation of CEH forming an increased number of particles of very high molecular weights. When this experiment was performed using adipose tissue a centrifugation step was included after incubation with apolipoprotein A-I to remove precipitated material (Khoo, Drevon and Steinberg (1980)). When the subsequent chromatography was carried out, virtually no activity was found in the void volume. However, only diacylglycerol hydrolase activity was measured. This result suggested that there may be increased aggregation upon treatment of the enzyme with apoprotein A-I.

Comparison of Fig. 3.8a with Figs. 3.8b and c showed that the activity of CEH was reduced by about 50% after incubation with hHDL or apolipoprotein A-I. Therefore the lower activity observed in the retarded peak could be due to inhibition by the apolipoprotein A-I present (not shown Fig. 3.8, for ^{125}I -labelled apolipoprotein A-I elution profile on Sepharose CL-4B, see Figs. 3.10a and b). It is possible that Khoo, Drevon and Steinberg (1980) may also have observed this inhibitory effect as all their results were expressed as percentage of peak fraction activity and not in absolute activity. The solubilisation effect had proved to be less effective in bovine adrenal cortex than in adipose tissue. However the inhibitory effect of hHDL and apolipoprotein A-I on CEH activity was interesting, hence it was decided to investigate this more fully to elucidate the mechanism.

3.5.2 Effect of hHDL and apolipoprotein A-I on CEH activity

Samples of an ammonium sulphate (0-40%) precipitate prepared from bovine adrenocortical cytosol were incubated with various amounts of hHDL to final hHDL protein:CEH protein ratios (mg:mg) of 0, 1:2, 1:1, 3:2 and 2:1. The mixtures were incubated at 37°C and aliquots assayed for CEH activity at 2 h and 4 h. A further sample of ammonium sulphate precipitate was incubated at 4°C as an additional control. Table 3.2 shows that incubation of the enzyme alone at 37°C was sufficient to cause a time-dependent loss of CEH activity. The loss was over 50% after 4 h and may be due to the action of endogenous phosphoprotein phosphatases that have relatively little divalent cation requirements or to the action of proteases. Therefore the decreased activity observed in Fig. 3.8b could be explained in these terms. However, addition of hHDL to the incubation greatly increased the inactivation. The inactivation was almost complete by 2 h and subsequent decreases in activity were probably due to the reasons stated previously. The incubation appeared to be maximal at an hHDL protein:ammonium sulphate protein ratio approaching 1:1.

HDL is composed of 50% protein and 50% lipid by weight and of the total lipid composition, about 40% is cholesterol ester and 20% is free cholesterol (Chapman (1980)). Therefore for every 1 mg of HDL protein there will be approximately 400 µg cholesterol ester and 200 µg free cholesterol. The amount of hHDL-derived free and esterified cholesterol present in each incubation, and the amount introduced into the CEH assay system is shown in Table 3.3. The concentration of cholesterol oleate in the substrate emulsion was 183 nmol/0.2 ml aliquot, and therefore 120 µg of labelled substrate was added to each assay. Furthermore, much of the hHDL derived cholesterol ester will be esterified to linoleic acid which is a better substrate for CEH (Gorban (1980)).

hHDL protein: CEH protein (mg:mg)	CEH Activity (pmol oleic acid produced.min ⁻¹ .mg protein ⁻¹)	
	2 h	4 h
0	1999 \pm 18	1471 \pm 30
1:2	109 \pm 10	35 \pm 3
1:1	90 \pm 6	27 \pm 1
3:2	370 \pm 36	236 \pm 28
2:1	297 \pm 31	173 \pm 16

Table 3.2. Inhibition of CEH activity by hHDL. Samples of 1 ml ammonium sulphate (0-40%) precipitate (1 mgml⁻¹) in 20 mM Tris-HCl buffer pH 7.4 at 37°C were incubated at 37°C with hHDL (30 mg protein.ml⁻¹) in 10 mM Tris-HCl buffer pH 7.0 containing 150 mM sodium chloride and 0.1 mM EDTA, at the ratios shown above. After 2 and 4 h, portions were withdrawn and the CEH activity measured. The CEH activity after incubation at 4°C for 4 h was 3283 \pm 61 pmol oleic acid produced.min⁻¹.mg protein⁻¹. The results are expressed as means \pm S.E.M. of triplicate assays.

hHDL protein: CEH protein (mg:mg)	Cholesterol Ester (μg)		Free Cholesterol (μg)	
	In Incubation	In CEH Assay	In Incubation	In CEH Assay
0	0	0	0	0
1:2	200	20	100	10
1:1	400	40	200	20
3:2	600	60	300	30
2:1	800	80	400	40

Table 3.3. The amount of hHDL-derived cholesterol ester and free cholesterol in each incubation tube (CEH + hHDL) and consequently the amount present in the CEH assays (total volume in each incubation tube was 1.07 ml and 100 μl was assayed).

In addition to substrate competition, the amounts of hHDL-derived free cholesterol may also result in product inhibition. How important these factors are considering the already high lipid content of similar CEH preparations from bovine adrenocortical cytosol (Wallat and Kunau (1976)) was difficult to estimate but it was possible that these factors contributed to the decreased CEH activity observed.

The protein moiety of hHDL consists of over 70% apolipoprotein A-I, 25% apolipoprotein A-II and small amounts of apolipoproteins C and E. These proteins not only function as structural components of the lipoprotein particle but also apolipoprotein A-I is an activator of lecithin:cholesterol acyl transferase (LCAT) and apolipoprotein C-II is an activator of lipoprotein lipase. Therefore it was possible that the main effector in hHDL that inhibits CEH was an apoprotein. This possibility was supported by the result observed in Fig. 3.8c where the presence of apolipoprotein A-I alone caused a reduction in CEH activity. In this case the incubation was performed at 4°C and so inactivation due to phosphoprotein phosphatase and protease activities would be reduced. To investigate the hypothesis that apolipoprotein A-I inhibits CEH, samples of an ammonium sulphate (0-40%) precipitate prepared from bovine adrenocortical cytosol were incubated with various amounts of purified apolipoprotein A-I, for up to 6 h at both 4°C and 37°C.

Table 3.4 shows that in the absence of apolipoprotein A-I the CEH activity decreased in a time and temperature-dependent manner. This suggests that this inactivation of CEH may be due to the activity of endogenous phosphoprotein phosphatases and proteases in the enzyme preparation. Addition of apolipoprotein A-I resulted in an immediate decrease in CEH activity of 75% and 85% at the 1:2 and 1:1 apolipoprotein A-I:ammonium sulphate (CEH) protein ratios (mg:mg), respectively.

Temp. (°C)	AI protein: CEH protein (mg:mg)	CEH Activity (pmol oleic acid produced.min ⁻¹ .mg protein ⁻¹)			
		0 h	2 h	4 h	6 h
4	0	2342 ± 61	1433 ± 89	1257 ± 19	1179 ± 92
	1:2	629 ± 51	367 ± 10	318 ± 31	338 ± 3
	1:1	395 ± 3	267 ± 31	282 ± 5	200 ± 4
37	0	2342 ± 61	1383 ± 12	849 ± 10	567 ± 15
	1:2	629 ± 51	263 ± 29	194 ± 25	235 ± 22

Table 3.4. Effect of apolipoprotein AI on the activity of CEH.

Samples of ammonium sulphate (0-40%) precipitate (1 mgml⁻¹) prepared from bovine adrenocortical cytosol in 20 mM Tris-HCl buffer pH 7.4 at 37°C or 4°C were incubated with apolipoprotein AI (0.5 mgml⁻¹ in 20 mM Tris-HCl buffer pH 7.4 at 37°C) in the various ratios and at the two temperatures shown. The CEH activity was determined at 2 h intervals. The results are expressed as means ± S.E.M. of triplicate assays.

The inhibition was increased slightly by 2 h but not significantly after this indicating that the inhibition was almost instantaneous on mixing. Small decreases with time or temperature probably reflects the additive effects of phosphoprotein phosphatase and protease activity described previously.

A further experiment was performed to investigate the kinetics of the deactivation. Samples of ammonium sulphate (0-40%) precipitate prepared from bovine adrenocortical cytosol were assayed for CEH activity in the presence of increasing amounts of apolipoprotein A-I. Other samples of enzyme were activated by preincubation with cAMP and ATP/Mg²⁺ before addition of apolipoprotein A-I. The results are shown in Fig. 3.9. The amounts of apolipoprotein A-I used in this experiment were considerably lower, in terms of both µg/assay and apolipoprotein A-I:enzyme protein ratio, than those used in the previous experiment. In this experiment a dose-dependent inhibition was seen with both the control (basal) and activated enzymes. In the case of the activated enzyme, the apolipoprotein A-I inactivation was more pronounced but it appeared that as the amount of apolipoprotein A-I increased the activity of CEH decreased to a similar level in both cases. Therefore as the inactivation was dependent on both the amount of apolipoprotein A-I and the phosphorylation state of the CEH, one possible explanation was that apolipoprotein A-I possessed phosphoprotein phosphatase activity and was acting by dephosphorylation of CEH. Another possibility was that apolipoprotein A-I may stimulate an endogenous phosphoprotein phosphatase. To investigate these hypotheses it was decided to investigate these possible enzymic activities of apolipoprotein A-I.

3.5.3 Investigation of possible phosphoprotein phosphatase activity of apolipoprotein A-I

To investigate the possibility of apolipoprotein A-I possessing phosphoprotein phosphatase activity, samples of ³²P-labelled phosphohistone

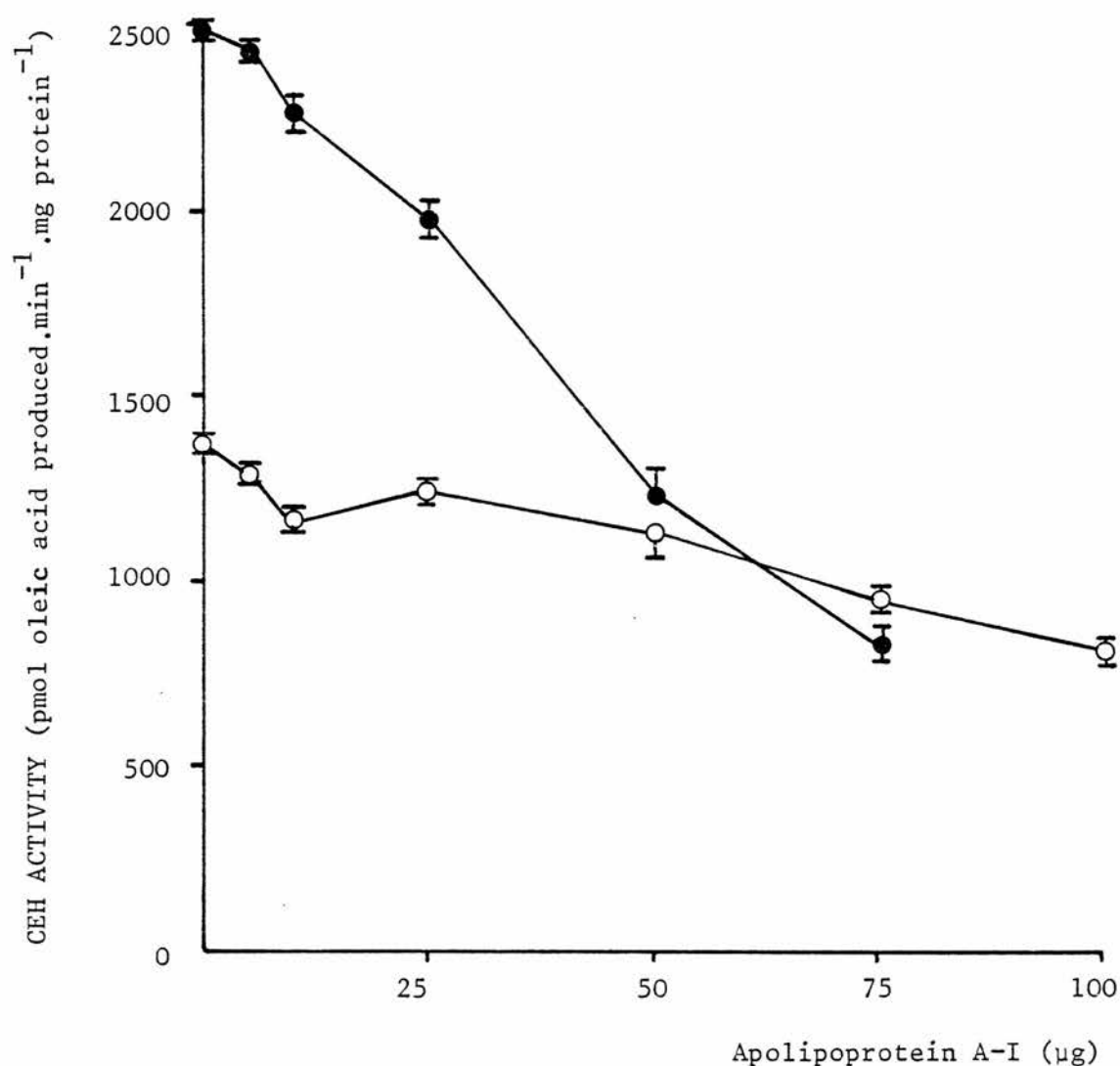


Fig. 3.9. Effect of increasing doses of apolipoprotein A-I on CEH activity. Samples of 100 μ l of an ammonium sulphate (0-40%) precipitate (2.58 mgml^{-1}) prepared from bovine adrenocortical cytosol in 20 mM Tris-HCl buffer pH 7.4 at 37°C containing 1 mM EDTA and 0.1 mM DTT were mixed with various amounts of apolipoprotein A-I (0.5 mgml^{-1} in 20 mM Tris-HCl buffer pH 7.4 at 37°C) and then assayed in triplicate for CEH activity. A parallel experiment was performed in which CEH was previously activated by incubation of the ammonium sulphate precipitate with ATP/Mg^{2+} and cAMP as described in the methods section. Each point is the mean \pm S.E.M. from two experiments.

O, Control enzyme; ●, Activated enzyme.

(50 μ l) containing 50 μ g protein and 0.2 μ Ci radioactivity were incubated for 30 min at 37°C with various amounts of apolipoprotein A-I. The release of radioactive phosphate was measured as described in the methods section. Table 3.5 shows that there was no significant difference between the ^{32}P content of the TCA supernatant after incubation with apolipoprotein A-I compared to control incubations with ^{32}P -labelled phosphohistone alone. This was also the case when the radioactivity in the protein pellet was measured. Inclusion of 5 mM Mg^{2+} in the incubations had no effect on the phosphoprotein phosphatase results.

Although phosphohistone is a commonly used artificial substrate for phosphoprotein phosphatases, there are examples in the literature of phosphoprotein phosphatases that have a low or no activity towards this substrate (Gratecos, Detwiler and Fischer (1974)). The substrate for the postulated phosphoprotein phosphatase activity of apolipoprotein A-I is phospho-CEH. Therefore, ^{32}P -labelled phospho-CEH was prepared from an ammonium sulphate (0-40%) precipitate from bovine adrenocortical cytosol by the procedure outlined in the methods section. This enzyme sample is impure and several proteins are known to be phosphorylated in this preparation. Samples of this preparation containing 125 μ g protein and 34 nCi radioactivity were incubated with various amounts of apolipoprotein A-I for 30 min at 37°C and the phosphoprotein phosphatase activity assayed as before. Table 3.6 shows there was no significant increase in the ^{32}P released after incubation with apolipoprotein A-I compared to the control incubation. The radioactivity in the protein pellet also showed no significant difference upon incubation with apolipoprotein A-I. No Mg^{2+} was included in these experiments but in the previous experiments where CEH activity was measured (see Fig. 3.9), no divalent cations were present and so any

Apolipoprotein AI added (μg)	^{32}P Content (pmol)	
	Supernatant	Pellet
0	115.5 \pm 0.3	677.2 \pm 17.6
25	106.1 \pm 3.2	676.0 \pm 20.1
50	105.5 \pm 2.7	679.4 \pm 22.4
100	109.9 \pm 1.3	681.2 \pm 19.8

Table 3.5. Incubation of ^{32}P -labelled phosphohistone with apolipoprotein AI. Various amounts of apolipoprotein AI (0.5 mgml^{-1}) in 20 mM Tris-HCl buffer pH 7.4 at 37°C were incubated for 30 min at 37°C with $50 \mu\text{l}$ ^{32}P -labelled phosphohistone (1 mgml^{-1} , $4.3 \mu\text{Ci.ml}^{-1}$) in 50 mM TES buffer pH 7.0. Phosphoprotein phosphatase activity was measured as described in the methods section. Results are means \pm S.E.M. of triplicate assays.

Apolipoprotein AI added (μg)	^{32}P Content (pmol)	
	Supernatant	Pellet
0	23.6 \pm 0.5	112.9 \pm 6.4
25	21.6 \pm 0.2	108.3 \pm 4.6
50	21.9 \pm 0.9	106.0 \pm 4.9
100	20.1 \pm 0.2	96.0 \pm 17.8

Table 3.6. Incubation of ^{32}P -labelled phosphoCEH preparation with apolipoprotein AI. Apolipoprotein AI was as described in the legend to Table 3.5. An ammonium sulphate (0-40%) precipitate from bovine adrenocortical cytosol was phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the methods section and desalted on Sephadex G-25 equilibrated in 20 mM Tris-HCl buffer pH 7.4 at 37°C containing 1 mM EDTA and 0.1 mM DTT. 100 μl samples of this preparation were incubated with various amounts of apolipoprotein AI for 30 min at 37°C and the phosphoprotein phosphatase activity measured as described in the methods section. Results are means \pm S.E.M. of triplicate assays.

putative phosphoprotein phosphatase activity would have to be independent of divalent cations. These results indicated that apolipoprotein A-I did not possess any detectable phosphoprotein phosphatase activity. It was possible that it was very specific towards phospho-CEH and the ^{32}P radioactivity released was, therefore, too small to be detected in such an impure enzyme preparation containing many phosphoprotein species. This would be a most unlikely conclusion as no phosphoprotein phosphatase had been found with such a high degree of specificity. However, a pure preparation of CEH would be required before this possibility could be completely excluded. This experiment also showed that apolipoprotein A-I did not stimulate an endogenous phosphoprotein phosphatase in the ammonium sulphate preparation.

3.5.4 Interaction of apolipoprotein A-I with cholesterol [$1\text{-}^{14}\text{C}$] oleate substrate emulsion

In HDL particles apolipoprotein A-I interacts with, and is in close association with, the other components of the particle. These components include a large amount of cholesterol ester, cholesterol and phospholipid and a small amount of triacylglycerol and other apolipoproteins (Chapman (1980)). Therefore it was likely that apolipoprotein A-I would interact with the cholesterol [$1\text{-}^{14}\text{C}$] oleate substrate emulsion forming an apolipoprotein A-I:cholesterol oleate complex. The formation of this complex may result in the cholesterol oleate being no longer freely available for hydrolysis by CEH. However, any such complex may have similar structure to HDL, and the lipids of HDL are available to lipase activity in the tissues that utilise HDL. It had also been shown that neutral CEH activity in adipose tissue cytosol can effectively hydrolyse the cholesterol esters in native rat plasma HDL (Khoo, Drevon and Steinberg (1979)). However the complexes formed in vitro may be sufficiently different to prevent effective hydrolysis, and so the

interaction between apolipoprotein A-I and the cholesterol oleate substrate emulsion was investigated.

The technique chosen to study the interaction was gel filtration chromatography. It was known that the cholesterol oleate substrate emulsion particles were eluted in the void volume of Sepharose CL-4B, and apolipoprotein A-I with a molecular weight of just over 28 000 should be maximally retained by this column and so be eluted much later. By using the cholesterol $[1-^{14}\text{C}]$ oleate labelled substrate emulsion and ^{125}I -labelled apolipoprotein A-I (see methods section) the elution profiles of both components were determined. In each interaction experiment two columns were run, one using labelled substrate emulsion and unlabelled apolipoprotein A-I and one with unlabelled substrate emulsion and labelled apolipoprotein A-I. One possible problem with this method was that, in the aqueous medium of the experiments, the apolipoprotein A-I may aggregate into large molecular weight particles that would also be excluded from the gel. Figs. 3.10a and b show that this did not occur, and whereas the cholesterol oleate substrate emulsion droplets were eluted in the void volume, the apolipoprotein A-I eluted at $2.4 \times V_o$. The A_{280} profile in Fig. 3.10a shows, firstly, scattering due to the emulsion particles in the void volume and secondly, the absorbance at about $2.4 \times V_o$ due to the bovine serum albumin present in the substrate emulsion. Therefore this technique was suitable for investigating the interaction between the substrate emulsion and apolipoprotein A-I.

When cholesterol oleate substrate emulsion was mixed with apolipoprotein A-I and the sample subjected to gel filtration on Sepharose CL-4B, the results obtained were identical to the two graphs obtained in Figs. 3.10a and b. Therefore no interaction between the two components was detected under these conditions. A major component of

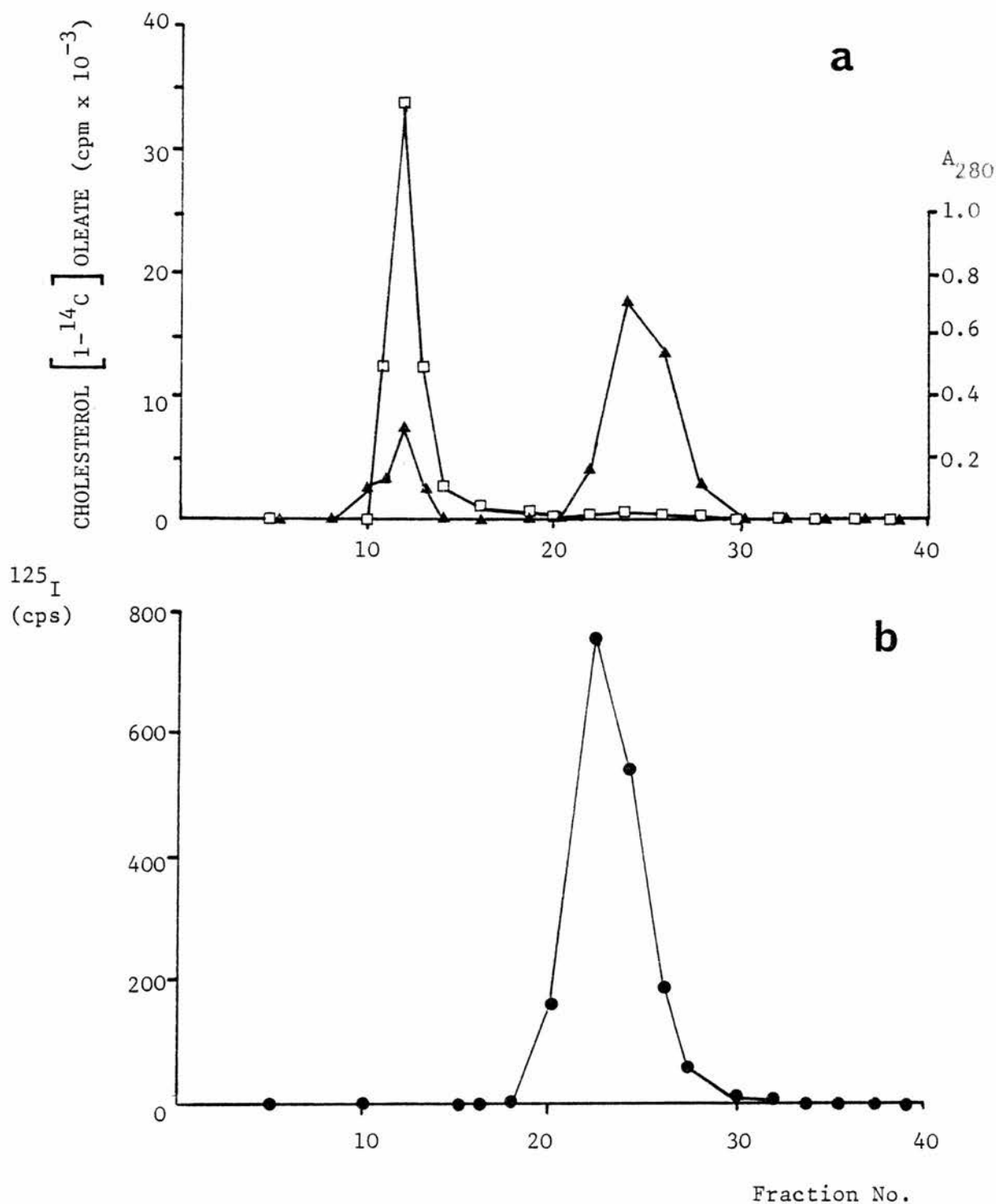


Fig. 3.10. Interaction of apolipoprotein A-I with cholesterol oleate substrate emulsion. ^{125}I -labelled apolipoprotein A-I (approximately $0.4 \mu\text{Ci} \cdot \mu\text{l}^{-1}$ in 50 mM potassium phosphate buffer pH 7.4) was prepared as described in the methods section. Unlabelled apolipoprotein A-I (1 mg ml^{-1}) was in 20 mM Tris-HCl buffer pH 7.4 at 4°C . Cholesterol $[1-^{14}\text{C}]$ oleate substrate emulsion contained 0.6 mg cholesterol oleate and 10^6 cpm per ml (see methods section) and the unlabelled emulsion was prepared identically except no radiolabel was included. The Sepharose CL-4B column ($2.5 \times 50 \text{ cm}$) was equilibrated and eluted with 20 mM Tris-HCl buffer pH 7.4 at 4°C .

a) 1 ml cholesterol $[1-^{14}\text{C}]$ oleate substrate emulsion was applied to column.

b) 1 ml ^{125}I -labelled apolipoprotein A-I solution (0.5 ml unlabelled apolipoprotein A-I, $25 \mu\text{l}$ ^{125}I -labelled apolipoprotein A-I in a total volume of 1 ml) was applied to column.

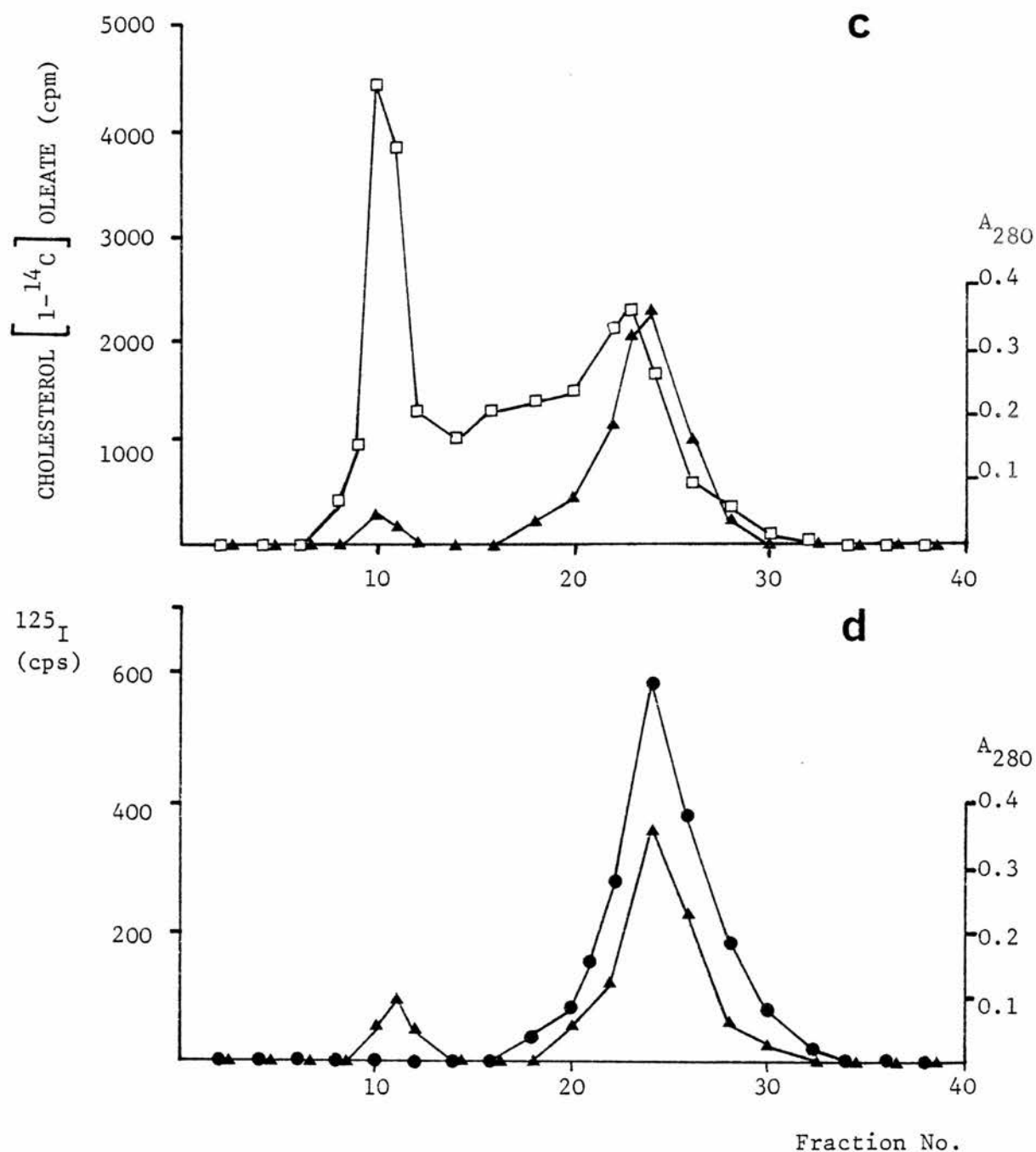


Fig. 3.10 contd.

c) 0.5 ml unlabelled apolipoprotein A-I was mixed with 0.5 ml cholesterol $[1-^{14}\text{C}]$ oleate substrate emulsion and 5 μl egg yolk lecithin (50 μg) and sonicated on a Rapidis 150, power 7 for 40s (4 x 10s) at 4°C, before application to column.

d) As c) except ^{125}I -labelled apolipoprotein and unlabelled cholesterol oleate substrate emulsion were used.

^{14}C was determined in 500 μl samples of the fractions by liquid scintillation counting and ^{125}I was determined in 50 μl samples of the fractions in a Wilj γ -counter.

\square , ^{14}C ; \bullet , ^{125}I ; \blacktriangle , A_{280} .

HDL and an important component of all lipoproteins is phospholipid which, due to its amphipathic nature, is essential for the stability and maintenance of all the lipoproteins (Lux, Hirz et al. (1972)). There have been extensive investigations into the association of apolipoproteins A-I and A-II with phospholipid complexes (Swaney (1980), Swaney and Chang (1980)). Therefore it was possible that phospholipid may be necessary for the formation of apolipoprotein A-I: cholesterol ester complexes. A further experiment was performed in which the cholesterol oleate substrate emulsion was sonicated with apolipoprotein A-I in the presence of egg yolk lecithin. Figs. 3.10 c and d show that the addition of phospholipid had an effect on the dissociation of the emulsion particles as there was cholesterol $[1-^{14}\text{C}]$ oleate present throughout the column fractions with a major peak at $2.3 \times V_0$. This effect was found to be dependent upon the ratios of phospholipid to apolipoprotein A-I and cholesterol oleate. In this experiment 50 μg phospholipid was mixed with 500 μg apolipoprotein A-I and 300 μg cholesterol oleate. When the phospholipid content was raised to 200 μg the effect of the dissociation was much reduced and with 400 μg phospholipid the effect was abolished completely. At these high phospholipid concentrations, the phospholipid may preferentially form micelles. Figs. 3.10c and d also show that the effect of sonication with phospholipid appeared to be primarily on the emulsion particles. That is, they were dispersed into apolipoprotein A-I/cholesterol oleate/phospholipid complexes of smaller size. The apolipoprotein A-I was not introduced into the larger emulsion particles, but there was some broadening of the apolipoprotein A-I peak, possibly indicating a larger spread of particle size. Sonication without phospholipid, gave only a small degree of dissociation of the emulsion particles.

These experiments showed that sonication in the presence of phospholipid resulted in the association of the cholesterol oleate contained in the substrate emulsion with apolipoprotein A-I. It was known that CEH preparations from bovine adrenocortical cytosol, similar to the ammonium sulphate preparation, were rich in phospholipids (Wallat and Kunau (1976)). Therefore it was possible that apolipoprotein A-I could interact with the cholesterol oleate substrate emulsion in the presence of the phospholipid contained in the CEH preparation. It was possible that the complexes formed as a result, contain cholesterol oleate in a central core or some other situation where it was not available to the CEH.

It has been shown recently that apolipoproteins A-I and A-II inhibit hepatic triglyceride lipase from human post-heparin plasma (Kubo, Matsuzawa et al. (1981)). Earlier work had shown that whole serum and purified HDL inhibited this enzyme when added to the assay (Kubo, Matsuzawa et al. (1980)). The concentrations of apolipoproteins A-I and A-II required to inhibit the enzyme activity were similar to those found in this study for inhibition of CEH activity. Concentrations of up to $240 \mu\text{g ml}^{-1}$ of A-I/A-II protein were added to the assay to give 50% inhibition of hepatic triglyceride lipase. This compares with the CEH inhibition in this case, where 100 μg and 50 μg of apolipoprotein A-I were required in the CEH assay (0.4 ml total volume) to give 50% inhibition of the enzyme in the basal and activated states respectively (Fig. 3.9). However Kubo, Matsuzawa et al. (1981) did not give any indication of the possible mechanism of this inhibition.

The finding that first HDL, and then purified apolipoprotein A-I inhibit CEH was similar to the situation with hepatic triglyceride lipase and like that system the mechanism of the inhibition is unclear. Preliminary work has shown that apolipoprotein A-I does not contain

any phosphoprotein phosphatase activity and does not stimulate endogenous phosphoprotein phosphatases in the CEH preparation. The apolipoprotein A-I may interact with the cholesterol oleate substrate emulsion in the presence of phospholipid contained in the CEH sample, and so block access of the CEH to the ester function of the substrate.

3.6 SUMMARY

1. Sodium cholate has been found to effectively solubilise CEH activity in ammonium sulphate precipitates prepared from bovine adrenocortical cytosol.
2. The cholate treatment inhibits the enzyme on long term exposure but glycerol can protect the enzyme from this inhibition.
3. However, cholate was not compatible with the use of ion-exchange techniques for the purification of the enzyme. Chromatography of CEH in cholate buffers leads to inactivation of the enzyme.
4. Human HDL and purified apolipoprotein A-I were not effective at solubilising the enzyme activity in ammonium sulphate precipitates.
5. Both hHDL and apolipoprotein A-I inhibited the activity of CEH.
6. The mechanism of inhibition by apolipoprotein A-I was investigated and discussed.

SECTION 4

PURIFICATION OF CHOLESTEROL ESTER HYDROLASE FROM BOVINE

ADRENOCORTICAL CYTOSOL II - HYDROPHOBIC INTERACTION

CHROMATOGRAPHY AND AFFINITY CHROMATOGRAPHY

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4.1 INTRODUCTION

Two major biochemical separation techniques had not been attempted previously with adrenocortical cytosolic CEH. These were hydrophobic interaction chromatography and affinity chromatography.

Although proteins had been separated previously on lipophilic ion-exchange resins (Weiss and Bücher (1970)) it is generally accepted that hydrophobic chromatography arose out of affinity chromatography when it was discovered that the alkyl spacer arms, used to link the ligand to the gel, could cause adsorption of the enzyme on their own (Er-el, Zaidenzaig and Shaltiel (1972)). This phenomenon was further studied (Yon (1972)) and found to be useful in the separation of proteins by their intrinsic differences in hydrophobicity (Yon (1974), Hjerten, Rosengren and Pahlman (1974), Shaltiel (1974), reviewed Hjerten (1973), Yon (1978)). There are now a great variety of different ligands that can be used for this purpose including alkyl, aminoalkyl and aryl derivatives of agarose which offer a wide range of hydrophobic and mixed hydrophobic/ionic affinities (Yon (1972), Yon (1974), Hjerten, Rosengren and Pahlman (1974)). Alteration of the chemical linkage between the ligand and the gel matrix (reviewed Yon (1978)), and of the gel matrix itself (Imamura and Horiuti (1979)) can alter the ability of the media to separate proteins by their intrinsic hydrophobic and hydrophobic/ionic differences.

The natural substrates for CEH in adrenal cortex are the long chain fatty acid esters of cholesterol (Beckett and Boyd (1975), Vahouny, Hodges and Treadwell (1979)). These compounds are almost totally insoluble in water and thus are hydrophobic, and so it is probable that the CEH enzyme molecule has an hydrophobic cleft at the active site, for the substrate molecule. The enzyme has also been shown to hydrolyse norcholesteryl oleate and pregnenol oleate (Beckett (1975)) and may also

catalyse the hydrolysis of triacylglycerols (Gorban and Boyd (1977), Pittman and Steinberg (1977)), hence it was possible that the fatty acid moiety was responsible for the binding of substrate to the active site of the enzyme. For these reasons it was thought that CEH would be an ideal enzyme to use in studies of protein separation by hydrophobic chromatography techniques. The aim of the first part of this work was to investigate the use of hydrophobic interaction chromatography in the purification of CEH from bovine adrenocortical cytosol.

CEH had been successfully solubilised from bovine adrenal cortex cytosol using cholate (see Section 3), and it was decided to use this preparation as the enzyme source for subsequent experiments. Although it was originally expected that hydrophobic chromatography would be useful in the purification of the most hydrophobic proteins, such as membrane proteins, it has been found that the detergents used to solubilise these proteins can interfere with the enzyme-ligand interactions (Yon (1978)). However, there were many examples of the successful use of hydrophobic chromatography in the purification of detergent solubilised proteins (Carson and Konigsberg (1981), reviewed Hjerten (1978)). Furthermore the purification of hepatic microsomal cytochrome P-450 had been reported using cholate solubilisation followed by hydrophobic chromatography on aminooctyl-Sepharose (Masuda-Mikawa, Fujii-Kuriyama et al. (1979)) or octyl-Sepharose (Beaune, Dansette et al. (1979)) columns, equilibrated in cholate-containing buffers.

Affinity chromatography was another potentially powerful technique for the purification of proteins, that had not been used previously with CEH from adrenocortical cytosol. The basic principle in affinity chromatography is to separate proteins from complex mixtures by

their binding to a ligand that is biospecific for a particular protein (Cuatrecasas (1970), reviewed Lowe (1977)). The ligand is usually covalently attached to a suitable gel matrix such as agarose or polyacrylamide (Cuatrecasas (1970)), although other supports such as glass (Weetal and Filbert (1974)) and plastic (Kondorosi, Nagy and Denes (1977)) have been used. This technique has been useful in the purification of enzymes with nucleotide (or nucleotide derived) cofactors, but as over 30% of all known enzymes can recognise the AMP moiety, the problem for each specific protein has been the precise structure of the nucleotide derivative used as the ligand (Lowe (1977)). Much work has been carried out using amino-acids, nucleic acids and sugars, and their respective derivatives, as ligands (reviewed Wilcheck and Jakoby (1974)). Lectins are also used widely (Sieber (1977)) and there are numerous applications of affinity chromatography using immunological procedures (Kondorosi, Nagy and Denes (1977), Weintraub (1970), Gospodarowicz (1972)).

With CEH there was only one known biospecific ligand, the substrate, with which to develop a true affinity column. The aim of the second part of this work was to investigate the use of affinity chromatography in the purification of CEH from bovine adrenocortical cytosol using cholesteryl ester as a biospecific ligand.

4.2 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Shaltiel hydrophobic chromatography kits (Er-el, Zaidenzaig and Shaltiel (1972)) were obtained from Miles Laboratories Ltd. These kits contained a series of 1 ml columns of agarose gel that had various lengths of either alkyl or ω -aminoalkyl chains covalently bound.

4.2.1 Application of enzyme

The columns were equilibrated with 300 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol and

0.1% (w/v) cholate. 200 μ l of cholate solubilised enzyme (see Section 3.3.4) in the same buffer was applied to the column and washed through with 2 ml of the column buffer. Aliquots of the eluant were assayed for CEH activity and the percentage of activity eluted was plotted against the chain length. It had been found that with most proteins, as the ligand chain length increased, in general the amount of protein bound increased (Shaltiel (1974)). Fig. 4.1 shows that this pattern was only found with the ω -aminoalkyl columns, whereas with the alkyl-agarose series the percentage activity eluted decreased, as expected, to hexyl-agarose and then increased with octyl- and decyl-agaroses. The reason for this was not known as no comparative study on the hydrolysis of different fatty acid esters of cholesterol by cytosolic adrenocortical CEH had been reported, except indirect studies by Beckett and Boyd (1975) and Vahouny, Hodges and Treadwell (1979) in which the differential hydrolysis of cholesterol esters of rat adrenal lipid droplets and cholesterol esters of rat adrenal cells was measured before and after subjecting the animal to hormonal stimulus such as ether stress (Beckett and Boyd (1975)) or challenging adrenal cells with dibutyryl cAMP (Vahouny, Hodges and Treadwell (1979)). The in vitro assay of CEH involves presenting the cholesterol ester substrate to the enzyme in a ethanolic emulsion. The technical difficulties in presenting esters of cholesterol with different fatty acid moieties such as stearate, palmitate, oleate and linoleate that have different chain lengths and degrees of saturation, to CEH under identical substrate emulsion conditions have prevented direct studies. Therefore it was not possible to speculate on the physiological significance, if any, of this observation.

A control column, with no ligand attached was included in each kit, and Fig. 4.1 shows that 87% of the CEH was eluted from the control column

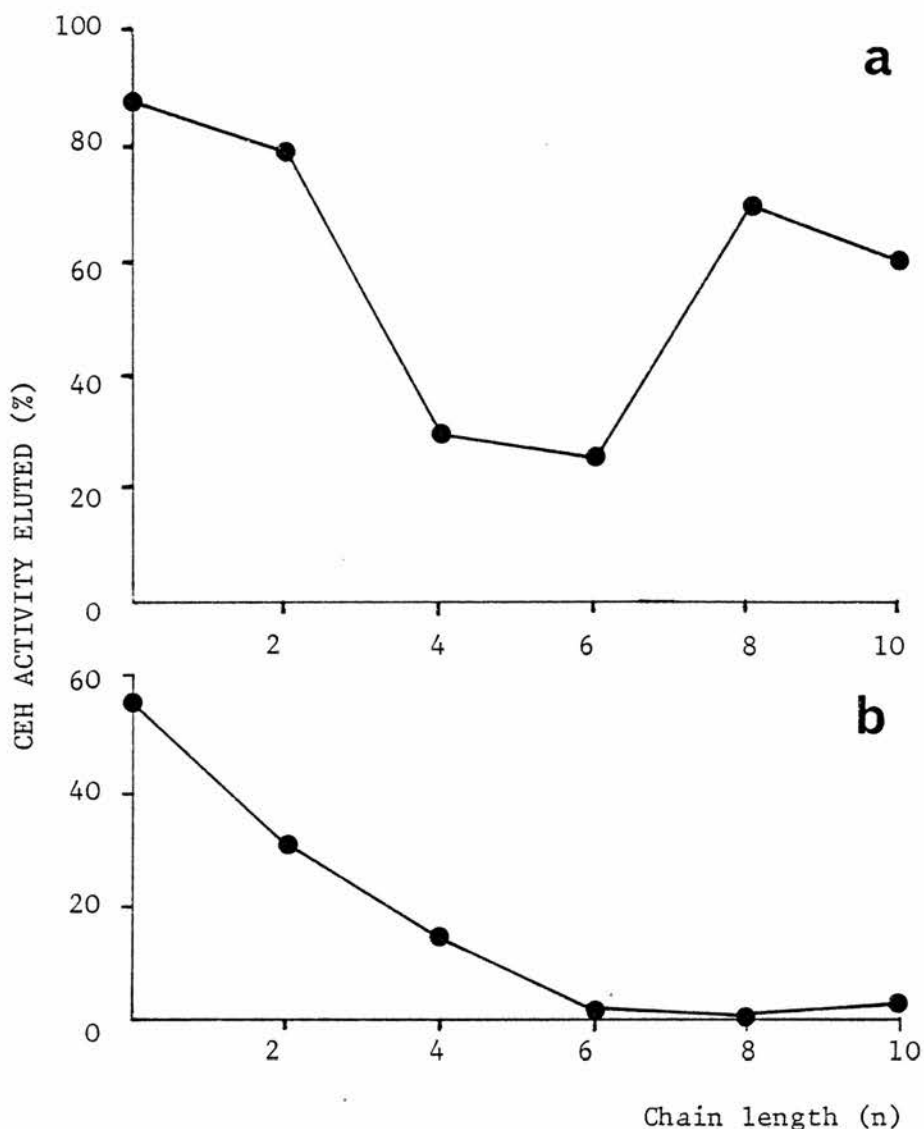


Fig. 4.1. Shaltiel hydrophobic chromatography test kits. 200 μ l cholate solubilised enzyme (4.8 mgml^{-1}) in 300 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol and 0.1% (w/v) cholate was applied to the columns, previously equilibrated with the sample buffer. The sample was washed through with 2 ml of the same buffer and the eluant assayed for CEH activity. A further 200 μ l sample of cholate solubilised enzyme was diluted with 2 ml buffer and assayed to give the control (100%) activity. The percentage of the control activity eluted was plotted against alkyl or ω -aminoalkyl chain length (n).

- a) Alkyl agarose columns;
- b) ω -aminoalkyl columns.

included in the alkyl agarose kit whereas only 56% was eluted from the apparently identical column included in the ω -aminoalkyl kit. Although this was difficult to explain these figures were compatible with the pattern obtained for each series and so it was not considered a major problem. Whereas the alkyl-agarose columns contain purely hydrophobic ligands the ω -aminoalkyl agarose series has a mixed function ligand containing both hydrophobic and ionic elements (Hjerten (1973)). This may introduce an additional, unwanted interaction between the matrix and the ionic detergent. Furthermore it was known that interactions between hydrophobic gels and detergents tend to interfere with the protein-adsorbant interactions (Yon (1978), Hjerten (1978)). However the extent of these ionic interactions should be relatively minor in the buffer system used, where the pH and relatively high ionic strength would reduce these forces. In both the alkyl series and aminoalkyl series, the ligand with 6 carbons in the chain proved to be the most effective at binding CEH and so these columns were chosen for further experiments on the elution of the bound activity from the gel. ω -Aminohexyl agarose was chosen in preference to the aminooctyl and aminodecyl derivatives as the binding forces should be the minimum required for complete retention of the enzyme.

4.2.2 Elution of CEH activity from hydrophobic columns

If hydrophobic chromatography was to be useful in the purification of CEH, it must yield an enzyme preparation of increased specific activity and also allow substantial recovery of the enzyme activity. This was investigated using the hexyl agarose and ω -aminohexyl agarose columns.

The columns were washed extensively with 1 M sodium chloride followed by treatment with ethanol, butanol, ethanol and distilled water (see Octyl Sepharose CL-4B, Phenyl Sepharose CL-4B, Pharmacia Fine

Chemicals, AB, Upsalla, Sweden (1976)) to remove all bound protein, and were then reequilibrated in the column buffer. A sample of 200 μ l cholate solubilised enzyme was applied and washed through with 2 ml column buffer as before. Various buffer systems were then applied to the columns and the percentage of activity found in the successive eluants was determined.

Table 4.1 shows that ω -aminohexyl agarose again bound almost all the CEH in the sample, but in this experiment the hexyl agarose also bound some 99% of the activity compared to only 76% bound in the first experiment (Fig. 4.1). However the results show that in general the enzyme was less tightly bound to the hexyl agarose column, as was expected. Despite this it was still tightly bound on both columns and none of the buffer systems investigated eluted more than 2.4% of the applied CEH activity. Although the protein content of each eluant was not measured in this experiment and thus the specific activity of the enzyme after each elution was not known, the overall recovery of the enzyme activity was sufficiently low that the use of hydrophobic chromatography under these conditions would be of little value in a CEH purification procedure. It was known that some proteins could bind almost irreversibly to hydrophobic ligands (Shaltiel (1974)) and this technique had been used for the non-covalent immobilisation of enzymes, in some cases, in their active state (Hofstee (1973)).

In these experiments a sample of cholate solubilised enzyme in relatively high ionic strength was applied to the columns and the CEH activity was found to be almost entirely bound in some cases. However all the elution conditions studied including lowering ionic strength, increasing detergent, changing the ions from those that are effective at increasing hydrophobic interactions (potassium and phosphate) to those that are much less effective (Tris and chloride) due to their relative

Buffer System	Elution Conditions	CEH in Eluant (% of Activity Applied)	
		hexyl- agarose	ω -aminohexyl- agarose
Sample Buffer	High Ionic Strength (I) Ions with high salting out effect	1.1	1.0
20 mM potassium phosphate buffer pH 7.4	reduced I	2.4	0
20 mM potassium phosphate buffer pH 7.4, 0.5% (w/v) cholate	reduced I increased detergent	1.3	0
20 mM potassium phosphate buffer pH 7.4. No cholate	reduced I reduced detergent	1.8	0
10 mM Tris-HCl buffer pH 7.4 at 4°C	reduced I different ions	0.6	0.8
10 mM Tris-HCl buffer pH 7.4 at 4°C, 0.5% (w/v) cholate	reduced I different ions increased detergent	0.5	1.5
10 mM Tris-HCl buffer pH 8.6 at 4°C	reduced I different ions increased pH	1.9	1.6

Table 4.1. Elution of bound CEH activity from hydrophobic chromatography columns. 200 μ l cholate solubilised enzyme (4.8 mgml⁻¹) in 300 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol and 0.1% (w/v) cholate was applied to the columns, previously equilibrated in the same buffer, and washed through with 2 ml of this buffer. Various elution buffers (4 ml) were applied to the columns sequentially and the amount of CEH activity in each eluant measured. All elution buffers were as for the sample buffer except for the changes stated above. Results are expressed as percentage activity applied, using 200 μ l cholate solubilised enzyme diluted with 2 ml sample buffer or 4 ml of each elution buffer as the appropriate 100% control.

salting-out and chaotropic effects, and increasing pH were all ineffective in eluting the enzyme activity. In some cases with more hydrophobic proteins, several bed volumes of elution buffer may be required to elute the protein, especially if a gradient is used (Simmonds and Yon (1976a&b)). However, in this case, the elution conditions were batch-wise, and each batch of buffer was four bed volumes giving a total elution volume in this experiment equivalent to 24 bed volumes. Therefore it was likely that if any CEH activity could be eluted by these conditions, it would be possible to detect this from this experiment.

With a technique such as hydrophobic chromatography there are many different methods that can be used in both the application of the protein preparation and also for the elution of the bound enzyme (see Hjerten (1973), Shaltiel (1974), Yon (1978)). For example, there are cases of application of the protein in low ionic strength buffer and elution with increasing ionic strength (Hjerten (1973)), although this may only be useful with the mixed hydrophobic/ionic columns such as the ω -aminoalkyl agaroses. There are many different methods for elution of the enzyme including changes in temperature and polarity of the eluant (Hjerten (1973), Shaltiel (1974)) in addition to those utilised here. Therefore after a study such as this it was not possible to state that hydrophobic interaction chromatography cannot, under any circumstances, be of value in the purification of CEH from bovine adrenocortical cytosol, as there are too many variables which could, theoretically, influence the performance of the system. It was possible that the ligands investigated here were too short, but as the binding of the enzyme to the column was not a problem, it seems unlikely that longer alkyl chains would be of any benefit. However using the cholate solubilised enzyme and the most commonly used

and, theoretically, the most appropriate methods with a range of both alkyl and ω -aminoalkyl agarose columns it was found that hydrophobic chromatography was of no benefit in the purification of this enzyme. For this reason it was decided not to pursue the investigation of this technique and to omit such a step from subsequent purifications of CEH from bovine adrenocortical cytosol.

4.3 AFFINITY CHROMATOGRAPHY

Affinity chromatography had not been used in any previous purification of CEH from adrenocortical cytosol. The main reason for this was that the only known biospecific ligands for the enzyme were the substrates, the cholesteryl esters of long chain fatty acids. There are three main problems associated with the use of these compounds in the preparation of a conventional affinity column:

- 1) These substrates are insoluble in water and so the coupling reactions would need to be carried out in an organic solvent in which cholesteryl esters are soluble and the gel matrix is stable (Cuatrecasas (1970)). Another approach might be to synthesise a more water-soluble derivative of the cholesterol ester, similar to the derivatives of steroids used in the preparation of affinity columns for steroid metabolising enzymes (Nicolas (1974), Benson, Surada et al. (1974)).
- 2) As these ligands are the substrates for the enzyme there is a possibility that the column may be hydrolysed by the enzyme as it is applied, although this should be substantially reduced by performing the experiment at low temperature. This possibility does raise an interesting feature of such a column, where the enzyme could be applied and bound to the column at low temperature and then eluted from the column simply by a rise in temperature. However this would be a rather expensive method to use if it were possible. Therefore it might be more useful to block the ester bond to prevent the hydrolysis

of the affinity column ligand. It would not be sufficient to use a cholesteryl ether of a long chain fatty alcohol as it was found that cholesterol ole yl ether was a poor inhibitor of the enzyme activity (Gorban (1980)) and so presumably the enzyme has low affinity for such compounds. Such a ligand with low biospecificity for CEH would operate simply as a hydrophobic interaction column.

3) Finally, cholesterol esters are fairly unreactive compounds with no reactive side groups other than the carbonyl of the ester bond.

Therefore the molecule would require to be activated at some position, for coupling to the gel matrix. Beckett (1975) found that the enzyme had low specificity towards the cholesterol side-chain moiety and so it might be possible to use a derivative with a reactive group on the cholesterol side-chain for this purpose. This would leave the rest of the molecule relatively free and flexible for binding to the CEH enzyme molecule.

However it was also possible to circumvent all these problems and indeed utilise one of them to make a simple and quick affinity column. It had been noted that it may be possible to non-covalently coat the surface of glass beads with a substance, particularly if it was "highly insoluble in water" (Cuatrecasas and Anfinsen (1971)). Although glass beads had been used quite widely as a support for affinity chromatography they have generally been porous, controlled-pore glass (CPG) beads with the ligand covalently bound (Weetal and Filbert (1974), Sieber (1977)). However there was a report where a ligand, in this case immunoglobulin, was coated non-covalently onto the surface of non-porous glass or plastic beads (Kondorosi, Nagy and Denes (1977)). Therefore it was decided to investigate the use of non-porous glass beads in the preparation of an affinity column for CEH.

4.3.1 The affinity chromatography column

Cholesterol oleate is a solid, water insoluble lipid at room temperature (m.p. 42-44°C). It is also the substrate used in most of the studies of cytosolic CEH, partly because it is the predominant unsaturated fatty acid ester of cholesterol found in the adrenal lipid droplets and partly because of the relative stability of this ester compared to esters of other long chain unsaturated fatty acids (Trzeciak and Boyd (1973), Beckett and Boyd (1975), Naghshineh, Treadwell et al. (1974), Wallat and Kunau (1976), Pittman and Steinberg (1977)). For these reasons cholesterol oleate was chosen as the ligand for the preparation of an affinity column.

Cholesterol oleate (0.5 g) was dissolved in diethyl ether (50 ml) and mixed with glass beads (3-4 mm diameter) which had previously been thoroughly cleaned by heating in chromic acid (65°C) for 1 h followed by extensive washing with distilled water and drying in a hot oven (90°C) for 2 h. The ether was evaporated under a light vacuum in a rotary evaporator (Blüchi Rotavapor-R) at room temperature, in a 1 litre round bottomed flask. At this stage the beads adhered to the wall of the flask and had an oily appearance. The flask was then removed from the rotary evaporator and shaken vigorously to dislodge the beads and this resulted in the coating of the beads having a white, semi-opaque and dry appearance. The beads were now able to move relatively freely over each other and did not adhere to the walls of the flask to any great extent. After the beads were dried under vacuum to remove the remaining diethyl ether, they were added to buffer. The thin coating of cholesterol oleate was found to be stable in aqueous buffer and did not float off from the surface of the beads.

The beads were carefully poured into a glass column (2.5 x 25 cm) containing some buffer, taking care to eliminate air bubbles trapped

in the system. The flow rate through the column was high and so all experiments were performed with a peristaltic pump to control the flow rate. Buffer (3 bed volumes) was slowly pumped through the column to remove loosely bound cholesterol oleate or any of the coating dislodged by the mechanical process of pouring the column. The affinity column was then ready for use in the following experiments.

4.3.2 Application of the CEH enzyme sample

For the initial experiments it was decided to use the ammonium sulphate (0-40%) precipitate prepared from bovine adrenocortical cytosol as the enzyme source as it was possible that the cholate contained in the cholate solubilised enzyme preparation would disrupt and probably solubilise the cholesterol oleate coating on the beads. It was found that applying the enzyme in simple buffers containing no glycerol at either low ionic strength (20 mM potassium phosphate buffer pH 7.4) or high ionic strength (20 mM potassium phosphate buffer pH 7.4 containing 0.3 M potassium chloride or 0.3 M potassium phosphate buffer pH 7.4) did not result in the CEH activity binding to the column. The elution profiles were identical to the one shown in Fig. 4.2a with a large peak of activity coeluting with the unadsorbed protein peak. Application of high ionic strength buffers after enzyme application in low ionic strength and vice versa, and inclusion of 1% (w/v) cholate in these elution buffers did not elute any activity, confirming that no CEH had been bound.

The buffer system was changed to Tris-HCl and the pH of the application buffer raised. Figs. 4.2a and b show that when the enzyme was applied in 50 mM Tris-HCl buffer pH 7.4 at 4°C the enzyme activity coeluted with the unadsorbed protein peak, but when the enzyme was applied in 50 mM Tris-HCl buffer pH 8.4 at 4°C, very little activity eluted with the unadsorbed protein. Tris-HCl buffer was chosen for

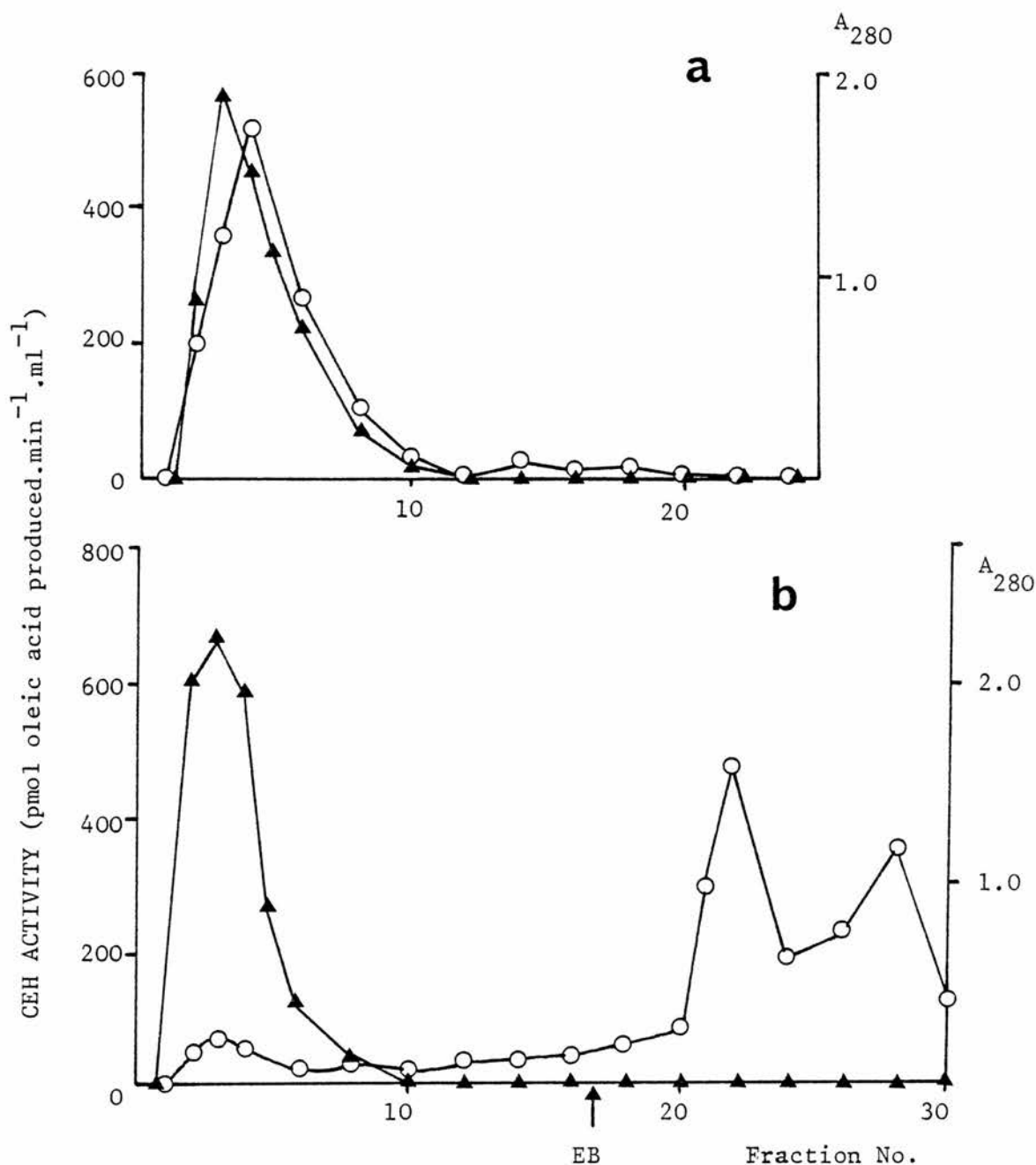


Fig. 4.2. Affinity chromatography on cholesterol oleate-coated glass beads. The cholesterol oleate coated-glass bead column (55 ml volume) was prepared and equilibrated with the appropriate buffer as described in the text. A 1 ml sample of an ammonium sulphate (0-40%) precipitate (19 mg ml^{-1}), prepared from bovine adrenocortical cytosol was dialysed against the appropriate buffer and applied to the column (10 ml.h^{-1}) and elution continued with the same buffer. Fractions of 4 ml were collected and CEH activity determined as described in the methods section.

a) Application of enzyme in 50 mM Tris-HCl buffer pH 7.4 at 4°C .

b) Application of enzyme in 50 mM Tris-HCl buffer pH 8.4 at 4°C .

At fraction 17, 10 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol and 0.25% (w/v) cholate was applied (EB).

○, CEH activity; ▲, A_{280} .

this experiment in order to obtain higher pH's, but this buffer is affected by temperature (Bates and Bower (1956)). This means that the pH in the CEH assay incubations may be altered as the pH in the Tris-HCl buffers will fall as the temperature rises from 4°C to 37°C (the assay temperature). Therefore Tris-HCl buffer with pH 7.4 or 8.4 at 4°C will decrease to approximately pH 6.5 and 7.5 at 37°C, respectively. However these changes will, themselves, be buffered by the 100 mM potassium phosphate buffer pH 7.4 in the cholesterol $[1-^{14}\text{C}]$ oleate substrate emulsion, which was added to the assays of the column fractions in equal amounts (200 μl column fraction plus 200 μl substrate emulsion). However to check this further, a series of Tris-HCl buffers at a range of pH's from 7.2-9.3 at 4°C were prepared and a concentrated ammonium sulphate (0-40%) precipitate from bovine adrenocortical cytosol was diluted 50 fold by each of these buffers. 200 μl aliquots of each of the diluted enzyme solutions had 200 μl cholesterol $[1-^{14}\text{C}]$ oleate substrate emulsion added (as in Fig. 4.2) and the CEH activity measured.

Fig. 4.3 shows that in 50 mM Tris-HCl buffer pH 8.4 the enzyme was only 65% as active as in the same buffer at pH 7.4. Therefore, although this buffer did lead to reduced CEH activity when assayed, the results show that pH inhibition cannot explain the total lack of activity eluting with the unadsorbed protein in Fig. 4.2b.

4.3.3 Elution of the bound CEH activity

Figs. 4.2a and b showed that CEH was bound to the column at pH 8.4 but not at pH 7.4. Therefore there was a possibility of eluting the bound enzyme by reducing the pH to 7.4. Fig. 4.2b shows that when 10 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol and 0.25% (w/v) cholate was applied to the column, the enzyme activity was eluted in possibly two peaks. No protein could be detected in these fractions, by A_{280} , or when selected fractions were

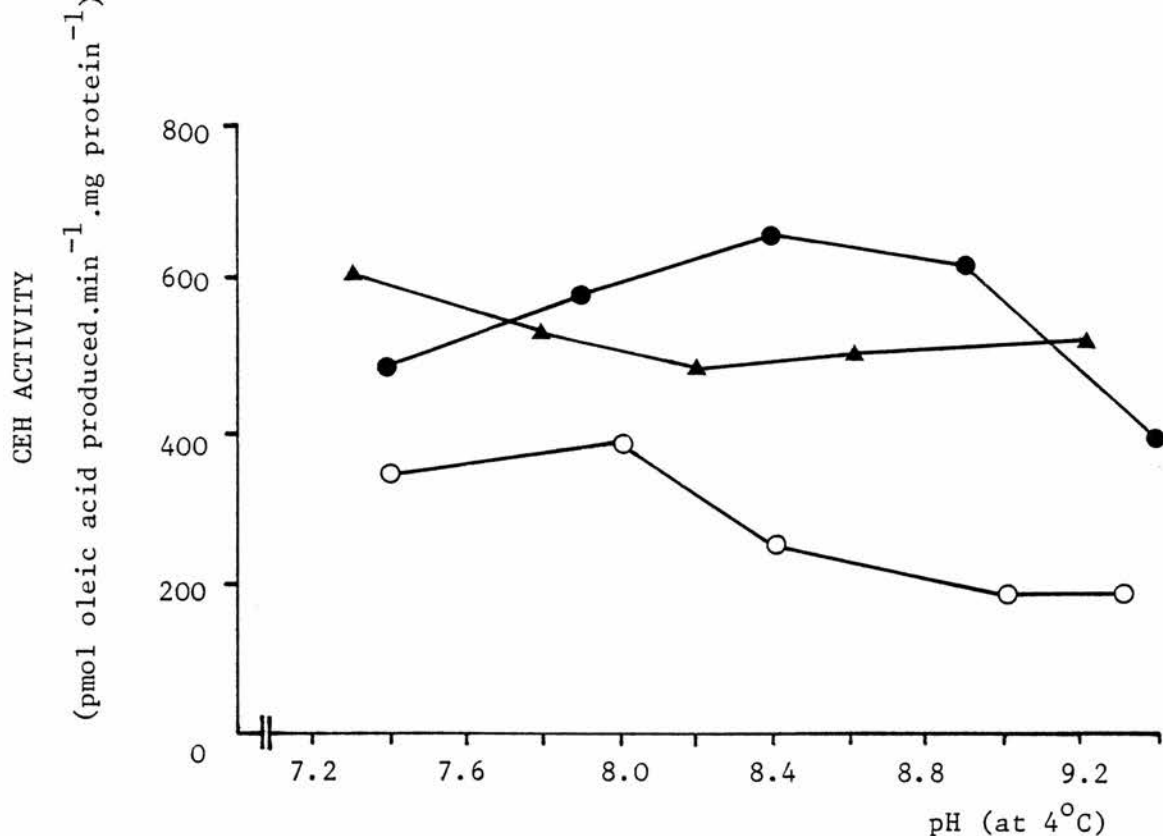


Fig. 4.3. CEH activity in Tris-HCl buffer systems at various pH's. A series of Tris-HCl buffer systems were prepared with various pH's at 4°C, as shown. An ammonium sulphate (0-40%) precipitate (50 mgml⁻¹) prepared from bovine adrenocortical cytosol was diluted to 1 mgml⁻¹ by each of the buffers at each pH. 200 µl of each diluted enzyme solution had 200 µl cholesterol [1-¹⁴C] oleate substrate emulsion added and CEH activity was assayed as described in the methods section. Each point is the mean of triplicate assays.

●, 10 mM Tris-HCl; ○, 50 mM Tris-HCl; ▲, 10 mM Tris-HCl containing 1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol and 0.25% (w/v) cholate.

assayed for protein by the method of Lowry, Rosebrough et al. (1951).

A further experiment was performed to study the relative contributions of reduced pH and cholate on the elution of the CEH activity.

A cholesterol oleate affinity column was prepared as before and 2 ml ammonium sulphate (0-40%) precipitate (19.2 mg ml^{-1}) was applied in 50 mM Tris-HCl buffer pH 8.4 at 4°C , and a series of buffers were applied successively to elute the enzyme activity.

Fig. 4.4 shows that application of twice as much protein had resulted in overloading of the column and some CEH activity eluting just after the unadsorbed protein peak, suggesting some retardation. In subsequent experiments it was found that application of more than 20 mg ammonium sulphate (0-40%) precipitate protein resulted in overloading, but no retardation of the overloaded activity was observed. The results shown in Fig. 4.4 also show that reduction of the pH to 7.4 was sufficient to elute some bound activity, but that inclusion of 0.25% (w/v) cholate in the buffer eluted a further batch of enzyme activity. In all subsequent experiments the bound activity was eluted by application of 10 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol and 0.25% (w/v) cholate.

4.3.4 The purified CEH enzyme

The exact nature of the interactions involved in the binding of the enzyme to this column were not known, and as elution was possible by methods such as changes in pH and inclusion of detergent in the buffer it may not be valid to regard this as true affinity chromatography. However, there are many examples of non-biospecific methods being used for elution of bound proteins from affinity columns (Cuatrecasas and Anfinsen (1971)).

When the affinity chromatography experiment was repeated several times the elution profiles obtained were similar to Fig. 4.2b.

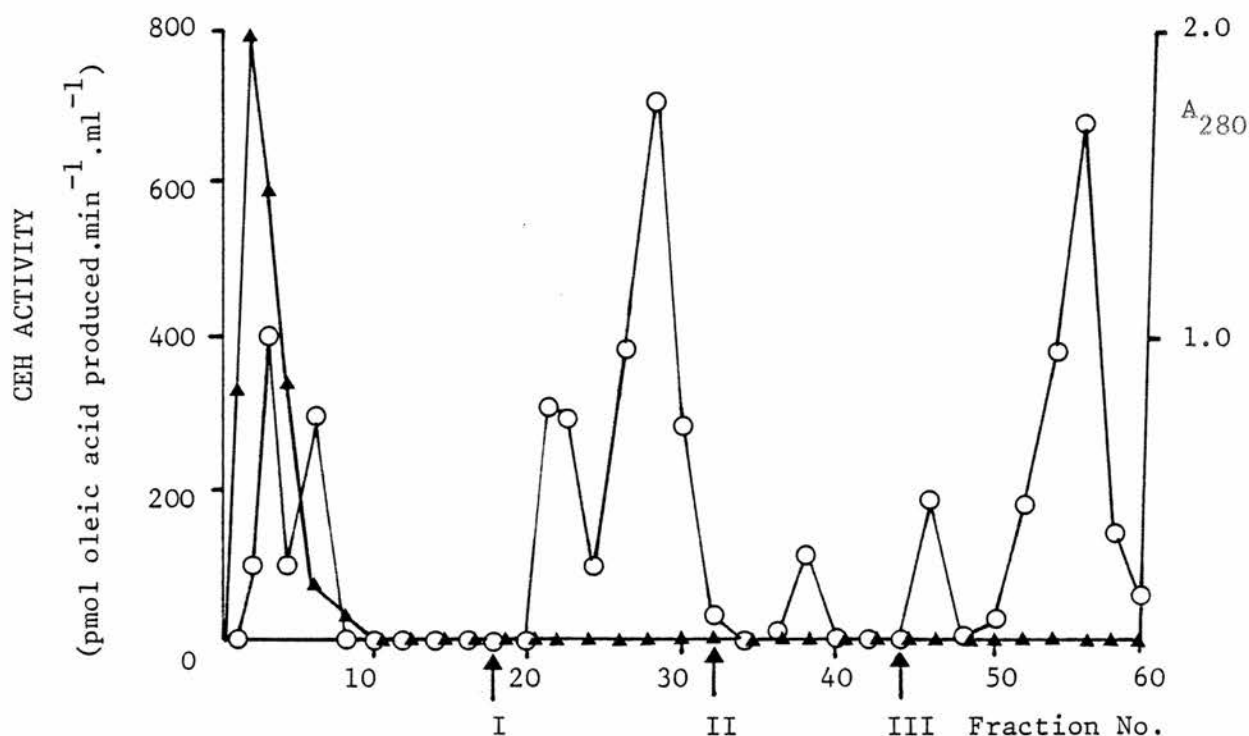


Fig. 4.4. Effect of pH and cholate on the elution of bound CEH from the cholesterol oleate affinity column. A cholesterol oleate affinity column (2.5 x 25 cm) was prepared as described in the text. 2 ml of an ammonium sulphate (0-40%) precipitate (19.2 mgml⁻¹) was applied in 50 mM Tris-HCl buffer pH 8.4 at 4°C and the column washed with 100 ml of this buffer. Two column volumes of the following buffers were applied as shown:

I - 10 mM Tris-HCl buffer pH 7.4 at 4°C;

II - 10 mM Tris-HCl buffer pH 7.4 at 4°C containing 0.05% (w/v) cholate;

III - 10 mM Tris-HCl buffer pH 7.4 at 4°C containing 0.25% (w/v) cholate.

○, CEH activity; ▲, A₂₈₀.

Unfortunately, all attempts to further study the purified CEH enzyme were unsuccessful. When the eluted enzyme activity was pooled and reassayed the activity was lost. Occasionally some activity did remain although much reduced, and was totally lost after storage overnight at either 4°C, -20°C or -70°C. If the pooled activity was immediately dialysed against buffers containing 50% (v/v) glycerol and 1 mM DTT to remove the cholate and stabilise the activity, there was no activity remaining in the dialysed preparation. Furthermore, no protein was detected in the fractions containing CEH when measured by A_{280} or by the method of Lowry, Rosebrough et al. (1951). When the pooled CEH activity was dialysed against volatile buffer, concentrated by lyophilisation and analysed by SDS/PAGE as described in the methods section, no protein was detected on the gels. At the time when these experiments were performed the results caused a certain amount of confusion, and a suitable explanation for them was difficult to produce. The elution profile shown in Fig. 4.2b was observed on 9 or 10 occasions in repeat experiments and there was no evidence for any problems with the CEH assay system as controls, in which buffer containing no enzyme was applied to the column, followed by elution buffers, did not result in any detectable enzyme activity. However the eluted CEH activity was impossible to maintain and, furthermore, no protein could be detected on polyacrylamide gels corresponding to the pooled CEH activity.

The only possible explanation that would fit this data was that the CEH enzyme in bovine adrenocortical cytosol was present at extremely low concentration. As the enzyme activity was easy to measure in the cytosol, the enzyme must have a high specific activity in the pure state. In the affinity chromatography experiments the activity was able to be measured initially in the eluted fractions as they were assayed within 1 h of elution, but because the amount of

enzyme protein was extremely small the activity was soon lost. The activity may be lost due to the enzyme adhering to the glass walls of the test tubes, especially as the enzyme is hydrophobic, but the high cholate: protein ratio will also be inhibitory (see Section 3.2). This could also explain why no protein was observed on polyacrylamide gels, as the staining methods would not detect protein below 2-3 μ g. The possibility that this was the explanation for these results was not fully appreciated at the time. However it was known that the affinity columns were of low capacity, as only 20 mg of protein could be applied without leakage of the CEH activity with the unbound protein. An attempt was made to increase the capacity of the column by using smaller beads. About 1 kg of glass beads (2-3 mm) were washed and prepared as described in Section 4.3.1 but it was not possible to coat these beads with cholesterol oleate. The coating was unstable and floated off the beads in aqueous buffers. The beads were lightly etched by rolling in a ball mill overnight, but again this procedure did not improve the stability of the coating and no successful results were obtained with these beads.

As it was not possible to maintain the purified CEH activity, or detect the protein in the pooled enzyme peak and as a column of greater capacity was not available this technique was not pursued further at that time, although it was attempted later (see Section 5.5.2).

4.4 SUMMARY

1. Hydrophobic interaction chromatography was investigated in the purification of CEH from bovine adrenocortical cytosol using Shaltiel test kits and cholate solubilised enzyme. With these columns and the conditions used it was found that the aminoalkyl derivatives of agarose bound the enzyme better than the corresponding alkyl derivatives.
2. Aminohexyl- and hexyl-derivatives were chosen for further studies but it was not possible to elute substantial quantities of the bound CEH from these columns.

3. The potential for hydrophobic interaction chromatography in the purification of CEH from adrenocortical cytosol was discussed.
4. A cholesterol oleate affinity chromatography column using glass beads as a support was developed.
5. CEH from bovine adrenocortical cytosol bound to the cholesterol oleate-coated glass beads at pH 8.4.
6. It was possible to elute CEH activity from the beads by reducing the pH and including cholate in the elution buffer.
7. The eluted activity was unstable and no further investigation was possible.
8. A possible reason for the instability of the eluted CEH activity was discussed.

PURIFICATION OF CHOLESTEROL ESTER HYDROLASE FROM BOVINE

ADRENOCORTICAL CYTOSOL III - NEW TECHNIQUES UTILISED IN THE

PURIFICATION OF HORMONE-SENSITIVE LIPASE FROM RAT ADIPOSE

TISSUE CYTOSOL

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5.1 INTRODUCTION

Triacylglycerol lipase in adipose tissue cytosol, the enzyme responsible for the cleavage of the first fatty-acid residue from triacylglycerols was found to be activated by adrenalin (Vaughan, Berger and Steinberg (1964)), and so was termed hormone-sensitive lipase (HSL). The activation was later discovered to be via a phosphorylation catalysed by cAMP-dependent protein kinase (Huttunen, Steinberg and Mayer (1970)). Following the discovery that CEH in adrenal cortex cytosol was also activated (Trzeciak and Boyd (1973)) via a cAMP-dependent phosphorylation (Trzeciak and Boyd (1974)), an activatable CEH activity was found in adipose tissue cytosol (Pittman and Khoo (1974), Pittman, Khoo and Steinberg (1975)). Subsequently an activatable triacylglycerol lipase was found in rat adrenal cytosol (Gorban and Boyd (1977), Pittman and Steinberg (1977)). In attempted purifications of either HSL from adipose tissue cytosol or CEH from adrenal cytosol, the two activities cofractionated (Pittman, Khoo and Steinberg (1975), Steinberg (1976), Pittman and Steinberg (1977), Gorban (1980)). A major problem that prevented the purification of HSL from adipose tissue, was the lipoprotein nature of the enzyme in the cytosol (Huttunen, Aquino and Steinberg (1970)) and a similar situation was found with the CEH in bovine adrenocortical cytosol (Beckett (1975), Wallat and Kunau (1976), Gorban (1980)). There had been much investigation and speculation on the possibility that these two enzyme activities were contained on the same enzyme protein (Pittman, Khoo and Steinberg (1975), Pittman and Steinberg (1977), Gorban and Boyd (1977), Gorban (1980), Yeaman, Cook and Lee (1980)).

Recently, in other laboratories, some success was obtained with the purification of HSL from rat and chicken adipose tissue. In both cases the cytosolic enzyme was solubilised by non-ionic detergent and

then subjected to ion-exchange chromatography. With chicken adipose tissue, Triton X-100 was used to solubilise the enzyme followed by hydroxylapatite, DEAE-cellulose and TEAE-cellulose chromatography in buffers containing Triton X-100, to give over 300-fold purification of the enzyme with 20% recovery (Berglund, Khoo and Steinberg (1979), Berglund, Khoo et al.(1980)). However with rat adipose tissue a purification for the HSL was reported, claiming a 2000-fold increase in specific activity from the tissue homogenate (Belfrage, Fredrikson et al.(1980), Nilsson, Stralfors et al.(1980)). This represented a large increase in the purity of the enzyme compared to previous work. However the details of the purification procedure were not reported until later (Fredrikson, Stalfors et al.(1981a)). In this purification scheme a pH 5.2 precipitate prepared from the cytosol was solubilised by non-ionic detergent followed by two unusual ion-exchange chromatography steps referred to as gradient sievorptive chromatography (Kirkegaard (1976)) and finally chromatography on "triacylglycerol-containing gel".

The initial stages of this purification up to, and including, the solubilisation by non-ionic detergent were similar to those used in other studies on HSL (Belfrage, Jergil et al.(1977), Berglund, Khoo and Steinberg (1979)) and also to the methods used in this study on CEH from bovine adrenocortical cytosol. However the gradient sievorptive chromatography procedure was a departure from the conventional ion-exchange chromatography that had been used previously with HSL in adipose tissue (Belfrage, Jergil et al.(1977), Berglund, Khoo and Steinberg (1979)) and also in this study with CEH from bovine adrenocortical cytosol. Furthermore, the final chromatography step on "triacylglycerol-containing gel" was the first reported use of an affinity technique for either HSL or CEH and was of particular interest due to the similarity to the affinity technique presented in this thesis.

Due to the similarities between HSL from adipose tissue and CEH from adrenal cortex, the novel techniques used, and the success of that purification, it was decided to investigate the use of some of these techniques in the purification of CEH from bovine adrenal cortex cytosol. It was hoped that this would not only provide a preparation of CEH of greater purity than obtained previously, but that it would also lead to a clearer understanding of the relationship between the HSL of adipose tissue and the CEH of adrenal cortex.

All procedures were carried out at 4°C unless otherwise stated.

5.2 INITIAL STAGES OF THE PURIFICATION

The first two stages of the purification were the same as those used in the previous purification schemes in this study (Sections 3 and 4). Adrenocortical tissue cytosol was prepared from about 30-40 bovine adrenal glands as described in the methods section. An ammonium sulphate (0-40%) precipitate was prepared essentially as described in Section 3.2. The collected precipitate was resuspended in 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 1 mM EDTA and 0.2 mM DTT in a total volume of 10 ml, and dialysed overnight at 4°C against 4.5 l of this buffer.

5.3 SOLUBILISATION OF ENZYME BY C₁₃E₁₂

Previous work in this study (see Section 3.4) had shown that the ionic detergent cholate, although effective at solubilising the CEH activity in the ammonium sulphate precipitate, interfered in ion-exchange chromatography procedures. The next two stages in the purification scheme were essentially ion-exchange techniques and so it was not possible to use cholate at these stages. Although it was possible to solubilise the enzyme with cholate and then exchange this detergent for a non-ionic detergent on hydroxylapatite, that process would involve a further and unnecessary chromatographic step.

Berol 058 (Berol Kemi AB, Stenungsund, Sweden) was the detergent used by Fredrikson, Stralfors et al. (1981a) in their procedure that resulted in a 2000-fold purification of HSL from rat adipose tissue. Although no direct evidence was given for the effectiveness of the solubilisation, this detergent was similar to Nonipol TD12 which had been shown to solubilise HSL in rat adipose tissue, as the solubilised enzyme eluted after the void volume on Sephadex G-200 superfine (Belfrage, Jergil et al. (1977)). Berol 058 is a polyoxyethylene monoether, designated $C_{13}E_{12}$ according to the general formula $C_nH_{2n+1}(OCH_2CH_2)_xOH$, abbreviated to C_nE_x (Tanford and Reynolds (1976)). It is a heterogeneous detergent containing $C_{10}E_{12}$ (<1.5%), $C_{12}E_{12}$ (54 %), $C_{14}E_{12}$ (44%) and $C_{16}E_{12}$ (<1.5%) and has an average molecular weight of 730 and a CMC of approximately 35 μM (20°C). It was decided to use this detergent to solubilise the CEH activity and so avoid the detergent exchange procedure necessary if cholate was used to solubilise the enzyme. However as the $C_{13}E_{12}$ detergent inhibits the enzyme activity, all enzyme samples must be appropriately diluted to non-inhibitory (approximately 0.001%) detergent concentrations. To the dialysed ammonium sulphate precipitate (10 ml), 2.86 g sucrose, 83.6 mg sodium chloride, 4.5 mg EDTA and 0.5 mg DTT were added to give final concentrations in the detergent-enzyme mixture of 13% (w/v) sucrose, 65 mM sodium chloride, 1 mM EDTA and 0.2 mM DTT. The enzyme solution was added in portions to 12 ml of 12% (w/v) $C_{13}E_{12}$ detergent in 30 mM Tris-HCl buffer pH 7.97 at 4°C. The detergent-enzyme solution was sonicated on a Rapidis 150 at power rating 7 for 2 min (8 x 15s) with the temperature maintained below 8°C. The solubilised enzyme was immediately applied to the first gradient sievorptive chromatography column.

5.4 GRADIENT SIEVORPTIVE CHROMATOGRAPHY

For maximal effect, fractionation procedures for cellular extracts should be as rapid and gentle as possible to minimise the loss of

biological activity. Sievorptive chromatography (Kirkegaard and Agee (1973)), the name given to systems that combine molecular sieve (gel filtration) and adsorption chromatography can be of great assistance in this task. There are three techniques available that use this combination. Ion filtration chromatography (Kirkegaard, Johnson and Bock (1972)), a process optimised for the separation of macromolecules of similar physical properties, intervent dilution chromatography (Kirkegaard and Agee (1973)) which utilises a solvent boundary to separate strongly interacting macromolecules, and gradient sievorptive chromatography (Kirkegaard (1973)). The theory and application of all three techniques is described in detail by Kirkegaard (1976). The following is a brief account of the principles and practice of gradient sievorptive chromatography.

During ion-exchange chromatography, proteins are fractionated by the inherent differences in their surface:charge distributions. Conventionally this has been achieved by applying the protein mixture at a pH at which most of the proteins present have a high net charge and will bind to a column matrix of opposite charge. The proteins are fractionated by application of a gradient, most commonly, of ionic strength. However if the pH is, say too high, in the case of cationic exchangers, the proteins will not be adsorbed and will be eluted and filtered according to the properties of the particular gel matrix. Between these two extremes of pH there will be a gradient of net charge on any protein and at some point this will result in the protein being fractionated by the combination of both processes (Fig. 5.1). In gradient sievorptive chromatography the conditions of pH and ionic strength are chosen exactly for the particular protein of interest so that it is in this narrow range of net charge whereas other proteins will either be adsorbed or eluted.

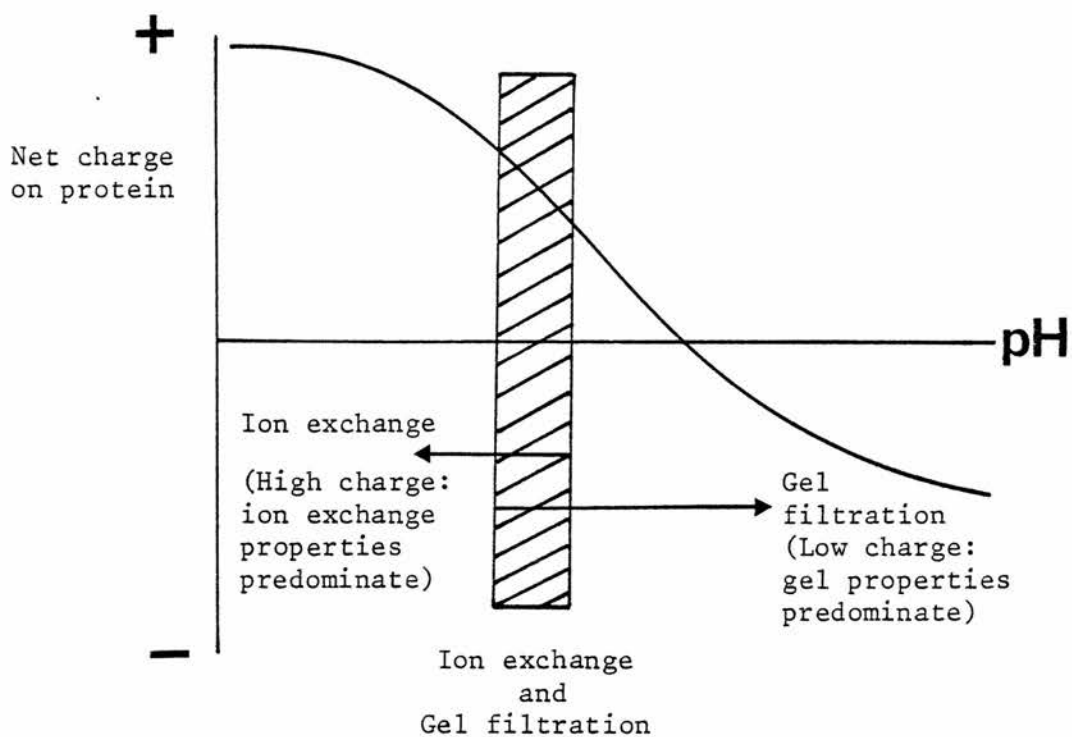


Fig. 5.1. The effect of pH on the net charge on a protein and its fractionation by a cation-exchange gel. At low pH the protein has a high net charge and will bind to the cation-exchange gel. At high pH the protein has low net charge and so the gel filtration properties of the gel predominate. Between these extremes there is a narrow band of pH where the net charge on the protein is such that neither property of the gel predominates and the protein is fractionated by both.

(Taken from Kirkegaard (1976)).

In practice, with the gels employed, the sieving properties are utilised to manipulate the behaviour of small molecules and the adsorptive properties are used to resolve the large molecules. Gradients of ionic strength are used to optimise the basic technique, and focus the protein of interest. The focusing effect of gradient sievorptive chromatography is summarised in Fig. 5.2.

Whereas normal ion-exchange is a passive process, this technique also has molecules projected forward by exclusion transport (see Fig. 5.2) and so has some characteristics of isoelectric focusing or density gradient centrifugation. Finally the buffer system must have the same charge as the gel and in the procedures described subsequently, QAE-Sephadex A-25 was used in conjunction with Tris-HCl buffers.

5.4.1 QAE-Sephadex A-25 I

The gradient sievorptive chromatography technique was applied utilising QAE-Sephadex A-25 as the ion-exchange gel. In this case a gradient was not applied to the column before the sample but was formed on the column by the sieving effects of the gel matrix on the ions in the buffers.

A QAE-Sephadex A-25 column (3.2 x 60 cm) was equilibrated with 10 volumes 0.1 M Tris-HCl pH 7.97 at 4°C (EB1), followed by 10 volumes 40 mM Tris-HCl buffer pH 7.97 at 4°C containing 15 mM sodium chloride (EB2) and 2 volumes 40 mM Tris-HCl buffer pH 7.97 at 4°C containing 15 mM sodium chloride, 0.2 mM EDTA, 0.2 mM DTT, 20% (v/v) glycerol and 0.2% (w/v) C₁₃E₁₂ (EB3). The solubilised enzyme (about 22 ml, 5% bed volume) was applied to the column followed by 35 ml of EB3 with the sodium chloride raised to 65 mM and containing 5% (w/v) sucrose. Elution was continued with EB3 with 65 mM sodium chloride (EB3/65) until all the unbound protein was eluted, and then a 65-1000 mM sodium chloride gradient in EB3 was applied to the column to elute the bound protein.

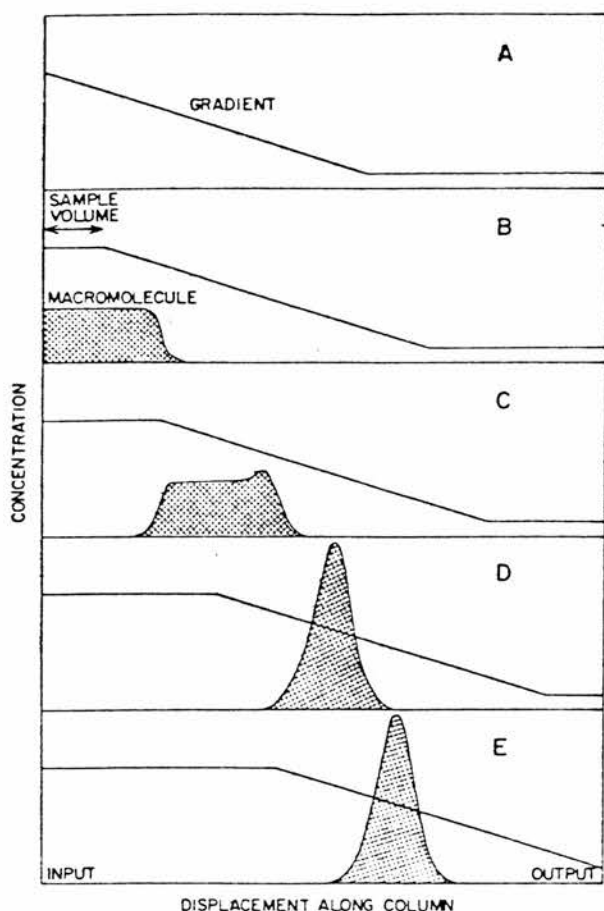


Fig. 5.2. Focusing properties of a sieving range gradient. Frames A-D show steps in the development of a sievortptive column utilising a gradient. The left-hand side of the figure represents the input of the column and the right-hand side the output. Frame A shows the gradient that is applied to the gel column before the addition of sample. The gradient is such that the applied macromolecule binds to the gel at the front of the gradient, but has no interaction with the resin at the back of the gradient. Frame B shows that the situation after a sample has been added to the column. In this illustration, the macromolecule travels twice as fast as the sample solvent; hence the macromolecular peak is twice as broad as the sample volume. It is further assumed that aside from the macromolecule, the sample has the same composition as the end point of the gradient. Frame C shows the situation after a volume of eluent equivalent to the sample volume has been added to the column. The leading edge of the macromolecule peak has reached the point in the gradient where adsorptive forces are beginning to slow the macromolecular migration rate to that of the gradient. Frames D and E, respectively, show the situation after three and four sample volumes of the eluent have been added to the column. After concentration the sample peak moves along the column at the same rate as the gradient migrates. The position along the gradient where the concentrated peak and the gradient move at the same rate depends upon the adsorptive properties of the macromolecule and of the gel.

(Taken from Kirkegaard (1973)).

CEH activity was eluted at 35-40 mM sodium chloride (Fig. 5.3). The active fractions were pooled as indicated and concentrated 10-fold by pressure ultrafiltration using an Amicon Diaflo PM 30 membrane. The sodium chloride concentration was kept at between 50 mM and 75 mM to prevent enzyme aggregation and adsorption to the membrane. The detergent was also concentrated during this procedure (Fredrikson, Stralfors et al. (1981a)).

5.4.2 QAE-Sephadex A-25 II

A column (2 x 96 cm) of QAE-Sephadex A-25 was equilibrated with 10 volumes EB1 followed by 10 volumes EB2 and 2 volumes of EB3 containing 17.5 mM sodium chloride. A 50 ml gradient of 17.5-50 mM sodium chloride in EB3 was applied to the column. The concentrated sample from the first QAE-Sephadex A-25 column was dialysed against EB3 containing 50 mM sodium chloride (EB3/50) and immediately applied to the column after the gradient. The column was eluted with EB3/50 and finally EB3 containing 100 mM sodium chloride was applied to the column to elute the bound protein.

CEH activity eluted at about 30-40 mM sodium chloride (Fig. 5.4). The active fractions were pooled as indicated and designated the CEH_{Q2} preparation.

5.5 AFFINITY CHROMATOGRAPHY

The greatest increase in specific activity (almost 14-fold) during the purification of HSL from rat adipose tissue reported by Fredrikson, Stralfors et al. (1981a) occurred at the final chromatography step on triacylglycerol-containing gel. This was obviously some form of affinity chromatography step, and so attempts were made to further purify the CEH_{Q2} preparation by an affinity chromatography method. Two different approaches were tried, one based upon the procedure described by Fredrikson, Stralfors et al. (1981a) and one based upon the procedures developed and described in Section 4.3 of this study.

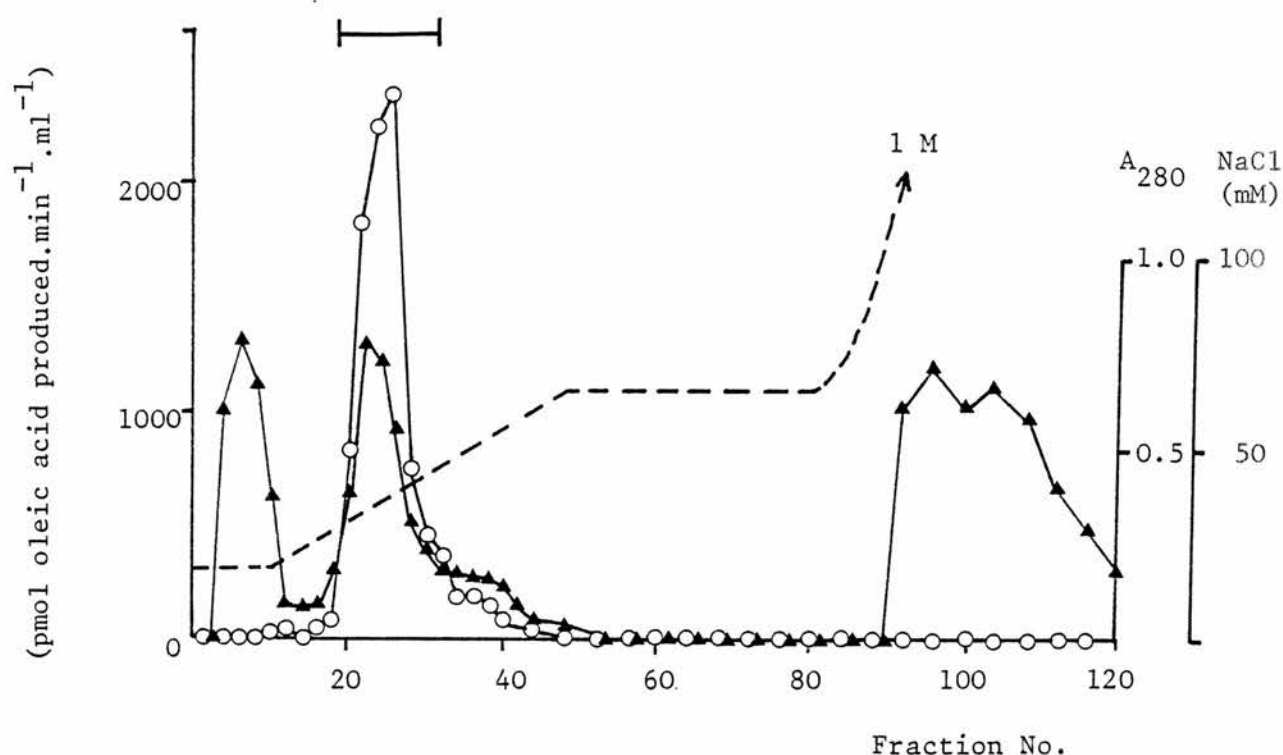


Fig. 5.3. Gradient sievorptive chromatography: QAE-Sephadex A-25 I. A QAE-Sephadex A-25 column (3.2 x 60 cm) was equilibrated as described in the text. The solubilised enzyme sample (22 ml) was applied, followed by 35 ml EB3/65 containing 5% (w/v) sucrose, and eluted with EB3/65. A 65-1000 mM sodium chloride gradient in EB3 was then applied. The column flow rate was 50 ml.h⁻¹. Glycerol containing 25 mM potassium dihydrogen phosphate and 0.2 mM DTT was added continuously to the column eluant at a rate of 27 ml.h⁻¹ to reduce the pH and raise the glycerol to 50% (v/v) and hence stabilise the eluted enzyme activity. Fractions of 12.8 ml were collected. C₁₃E₁₂ inhibits CEH activity (see Section 5.3) and so samples were diluted to reduce the detergent concentration for assay: 5 µl fraction had 95 µl 0.02% (w/v) bovine serum albumin in 20 mM potassium phosphate buffer added, followed by 100 µl cholesterol [1-¹⁴C]oleate substrate emulsion, and the assay completed as described in the methods section. O, CEH activity; ▲, A₂₈₀; --- Sodium chloride concentration; —, Fractions pooled.

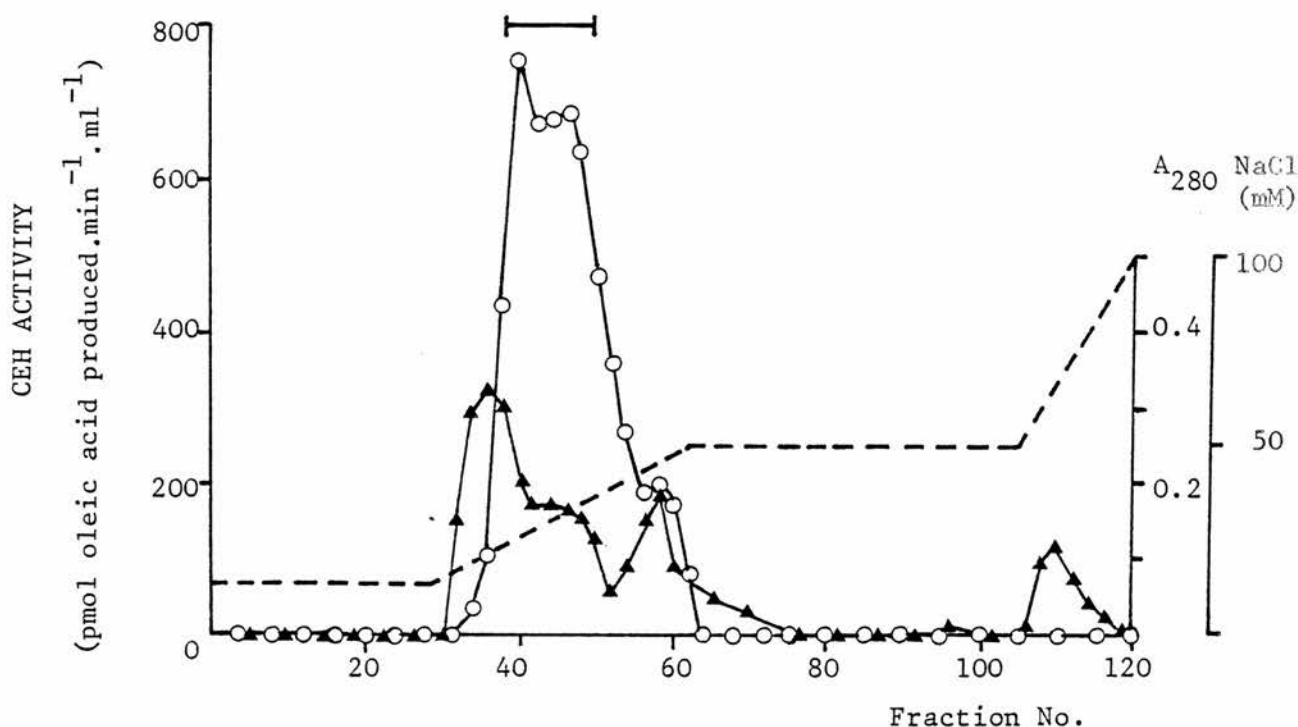


Fig. 5.4. Gradient sievorptive chromatography: QAE-Sephadex A-25 II. A QAE-Sephadex A-25 column (2 x 96 cm) was equilibrated as described in the text. The pooled and dialysed enzyme sample was applied to the column immediately after a 17.5-50 mM sodium chloride gradient (50 ml total volume) in EB3. Elution was continued with EB3/50. The adsorbed proteins were eluted with 100 mM sodium chloride in EB3. The column flow rate was 30 ml.h⁻¹ and acid glycerol was continuously added to the eluant at a rate of 17.5 ml.h⁻¹. Fractions of 9.5 ml were collected and assayed as described in the legend to Fig. 5.3.

○, CEH activity; ▲, A₂₈₀; ---, Sodium chloride concentration; —, Fractions pooled.

5.5.1 Affinity chromatography on cholesterol oleate-containing Ultrogel AcA34

The triacylglycerol-containing gel used in the purification of HSL from rat adipose tissue was pre-1977 Ultrogel AcA34 (LKB, Stockholm, Sweden). It was discovered that batches of this gel produced before 1977 contained approximately 0.3 mg of soyabean oil triacylglycerol per millilitre of wet gel. This was a consequence of the manufacturing procedure, but this procedure was changed by the manufacturers during 1977 and batches of the gel after that date cannot be used for this purpose. No pre-1977 Ultrogel AcA34 was available for this study, however this was not a major problem because a cholesterol ester affinity column was required, as the relationship between HSL and CEH was not fully understood.

To introduce cholesterol ester into the Ultrogel AcA34 a modification of the method suggested by Fredrikson, Stralfors et al. (1981b) was used. The main problem in this procedure was the insolubility of cholesterol ester in aqueous solutions. However, Ultrogel AcA34 is a molecular sieve (gel permeation) type of gel containing 4% agarose and 3% polyacrylamide and is similar to Sephacryl S-200, which is relatively stable in a number of organic solvents (Gel Filtration Booklet, Pharmacia Fine Chemicals). Cholesterol oleate was the cholesterol ester chosen for the ligand, as it is the predominant ester in adrenal lipid droplets and is more stable than other cholesteryl esters of long chain unsaturated fatty acids and has been used as the substrate in most of the studies on cytosolic CEH (Trzeciak and Boyd (1974), Beckett and Boyd (1977), Naghshineh, Treadwell et al. (1974), Wallat and Kunau (1976), Pittman and Steinberg (1977)).

Cholesterol oleate (10 g per 100 ml of gel) was suspended in 75% (v/v) ethanol containing 0.1% (w/v) sodium azide, and heated to 45°C.

This was added to Ultrogel AcA34, that had previously been washed in water followed by 75% (v/v) ethanol containing 0.1% (w/v) sodium azide. The mixture was stirred for 2 h at 45°C (cholesterol oleate m.p. ~42-44°C) and then transferred to a warm room (30°C) and stirred slowly overnight. The gel was then poured into 10 volumes of 1 M sodium chloride and washed extensively to remove the large quantities of excess lipid. The gel was then ready for use.

The $C_{13}E_{12}$ detergent inhibits the adsorption process of HSL on the triacylglycerol-containing gel, consequently the column was equilibrated in C_8E_6 detergent (Fredrikson, Stralfors et al. (1981a)). The reason for the different behaviour of the two detergents was not known, although the difference in critical micellar concentration was suggested as a possible explanation. The critical micellar concentration of C_8E_6 is 10 mM (Fredrikson, Stralfors et al. (1981a)) which is 100-fold higher than $C_{13}E_{12}$ (Helenius, McCaslin et al. (1979)). It is unusual for a non-ionic detergent to have such a high critical micellar concentration and it is similar to the critical micellar concentrations for ionic detergents, including sodium cholate (13-15 mM) (Helenius, McCaslin et al. (1979)). Therefore as C_8E_6 was not commercially available, and as much of the previous work in this study investigated the effect of cholate on the enzyme, it was decided to use cholate as the detergent in the affinity column.

A column (3.5 x 50 cm) of cholesterol oleate-containing gel was washed with 10 volumes of 1 M sodium chloride followed by 2 volumes of 5 mM potassium phosphate buffer pH 7.4 containing 30% (v/v) glycerol, 0.2 mM DTT, 0.15 M sodium chloride and 0.2% (w/v) $C_{13}E_{12}$ and finally equilibrated in 4 volumes of the same buffer with 0.1% (w/v) cholate replacing the $C_{13}E_{12}$. A sample of CEH_{Q2} preparation (2 mg) was dialysed against the column buffer, applied to the column and eluted with the same buffer. The results are shown on Fig. 5.5.

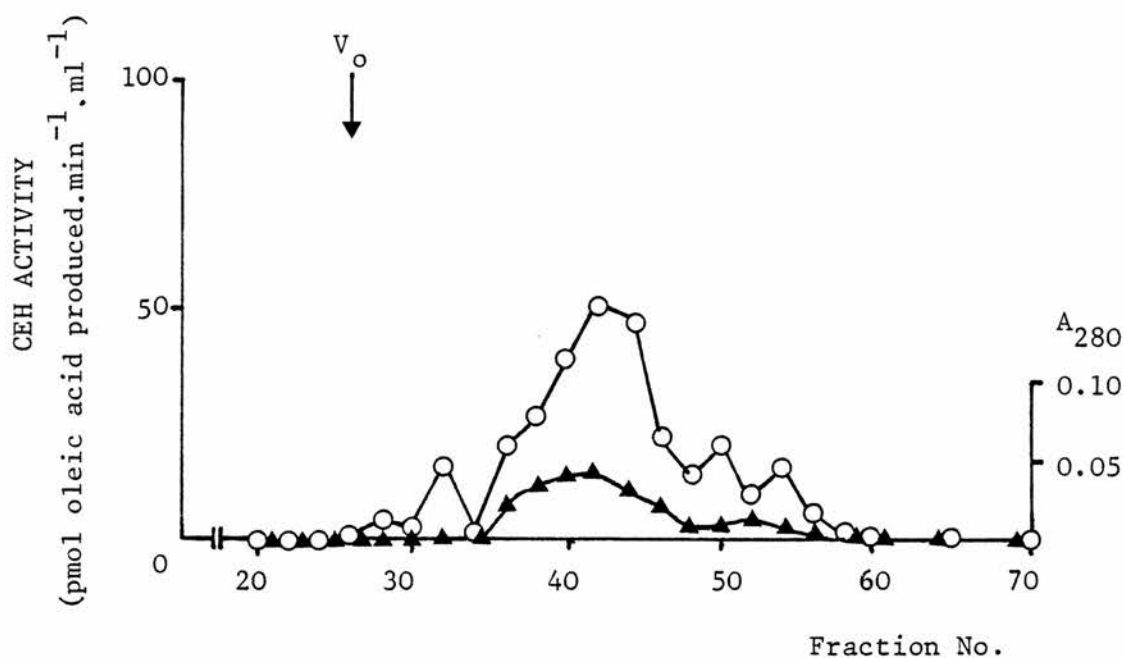


Fig. 5.5. Affinity chromatography on cholesterol oleate-containing Ultrogel AcA34. A cholesterol oleate-containing Ultrogel AcA34 column (3.5 x 50 cm) was equilibrated as described in the text and 10 ml concentrated CEH_{Q2} preparation (2 mg), previously dialysed against the column buffer, was applied and eluted with the same buffer. The fractions were assayed as described in the legend to Table 5.1.

○, CEH activity; ▲, A₂₈₀.

The protein was just detectable by A_{280} measurements and the CEH activities measured were extremely low, representing a maximum of only 20 cpm above background (67 cpm). When the peak fraction was assayed for specific activity there was a 4-fold decrease compared to the CEH_{Q2} preparation (see Table 5.1). The coelution of the activity and the protein shows that the affinity column did not work under these conditions. The reasons for this failure were not certain but it may be that the incorporation of cholesterol oleate into the gel was not successful. However the mechanism involved in the adsorption of HSL to the triacylglycerol-containing gel was not clear. Although affinity interaction was involved, non-specific hydrophobic and polar interactions were probably also part of the mechanism because although triacylglycerol was essential for adsorption, the eluted activity was only 50% HSL protein (Fredrikson, Stralfors *et al.* (1981a)). Therefore it was also possible that cholate could interfere with some of these interactions and so prevent any purification. It was probable that the low activity was due to the cholate induced deactivation observed in Section 3.4.

5.5.2 Affinity chromatography on cholesterol oleate-coated glass beads

Glass beads (3-4 mm) were washed, and coated with cholesterol oleate as described in Section 4.3.1. The beads were packed in a column (1.5 x 60 cm) and equilibrated with 50 mM Tris-HCl buffer pH 8.4 at 4°C containing 0.2 mM DTT, 20% (v/v) glycerol and 0.02% (w/v) cholate. A sample of enzyme after the first QAE-Sephadex chromatography step was concentrated and dialysed for 3 h against the column buffer, applied to the column and eluted with this buffer. The results are shown in Fig. 5.6.

This column was not successful as the enzyme activity was not retarded, and eluted with the unadsorbed protein. This result was not entirely surprising as circumstances dictated that the column be run in

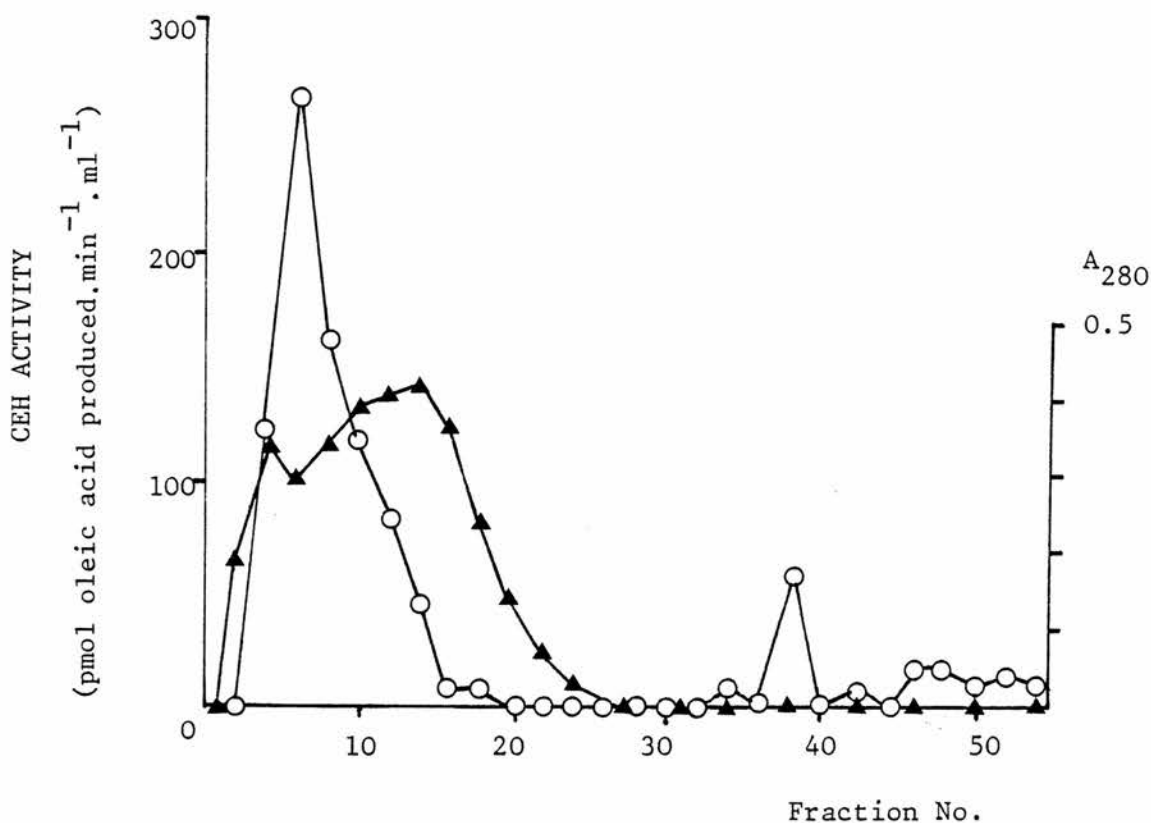


Fig. 5.6. Affinity chromatography on cholesterol oleate-coated glass beads. A sample (14 ml) of concentrated enzyme from the first QAE-Sephadex chromatography step was dialysed against 50 mM Tris-HCl buffer pH 8.4 at 4°C containing 0.2 mM DTT, 20% (v/v) glycerol and 0.02% (w/v) cholate. The dialysed sample was applied to a column (1.5 x 60 cm) of cholesterol oleate-coated glass beads (see Section 4.3.1) equilibrated and eluted with the sample buffer. The flow rate was 10 ml.h⁻¹ and acid glycerol was added to the fractions at a rate of 5.4 ml.h⁻¹. Fractions of 2.5 ml were collected and assayed for CEH activity as described in the legend to Fig. 5.3.

○, CEH activity; ▲, A₂₈₀.

a different set of conditions to those found to be successful in Section 4.3. This was because the enzyme was unstable and inactivated if the detergent was removed and so the column had to be run in detergent. As relatively high detergent was known to inhibit the binding to this column (see Section 4.3.3) it was decided to use this column in a similar way to the cholesterol oleate-containing gel. In the purification of HSL from rat adipose tissue the enzyme did not bind completely to the affinity gel but was retarded and eluted after the main protein peak, and so a long column was used (Fredrikson, Stralfors et al. (1981a)). Therefore it was hoped that it might be possible to use the glass bead column in a similar way (i.e. simply a different support for the ligand) and so the column was equilibrated in a low concentration of cholate plus glycerol and DTT to protect the enzyme. However the results show that the affinity column did not operate in this way and no retardation of the CEH was seen.

5.6 THE OVERALL PURIFICATION

A representative purification is summarised in Table 5.1. The ammonium sulphate (0-40%) precipitate was again shown to be an effective early batch-wise step. The enzyme activity in the $C_{13}E_{12}$ solubilised ammonium sulphate precipitate could not be measured accurately due to the high detergent concentration. Both the gradient sievorptive chromatography steps on QAE-Sephadex A-25 were successful and gave substantial increases in specific activity. However the affinity chromatography step on cholesterol-oleate containing Ultrogel AcA34 was not successful, resulting in no purification (Fig. 5.5) and substantial deactivation of the enzyme.

The procedure, up to the CEH_{Q2} preparation, gave a recovery from 19-38% with a purification of 122-162 fold (range of 4 purifications). The specific activity of the CEH_{Q2} preparation was on average 59 nmol

Step	Total Protein (mg)	CEH Activity		Purification (fold)	Recovery (%)
		Specific (units.mg protein ⁻¹)	Total (units)		
105 000 x g Supernatant	1659	0.36	602	-	100
Ammonium Sulphate (0-40%) precipitate	248	2.25	559	6.2	93
Solubilisation by C ₁₃ E ₁₂	248	N.D.	N.D.	-	-
QAE-Sephadex A-25 I	30.4	11.55	351	31.8	58
QAE-Sephadex A-25 II	3.6	55.00	198	151.5	33
Affinity chromatography on cholesterol oleate- containing gel	1.0	12.88	12.9	-	N.D.

Table 5.1. Summary of purification procedure. The enzyme was purified from bovine adrenocortical cytosol, prepared from about 30 adrenals as described in the methods section. The CEH activity was measured after dilution of the samples to non-inhibitory detergent concentrations (see Section 5.3): 5 µl enzyme preparation had 195 µl 0.02% (w/v) BSA in 20 mM potassium phosphate buffer pH 7.4 added, followed by 200 µl cholesterol [1-¹⁴C] oleate substrate emulsion, and was assayed as described in the methods section. Protein was measured by the method of Bradford (1976) with the appropriate calibration curves for samples with and without detergent. [N.B. The affinity chromatography step was known to have a low capacity (Fredrikson, Stralfors *et al.* (1981a)) and so only half the CEH_{Q2} preparation was chromatographed. Due to this problem and enzyme inactivation - recovery was not calculated.] Enzyme activity units are nmol oleic acid produced.min⁻¹.

oleic acid produced, $\text{min}^{-1} \cdot \text{mg protein}^{-1}$. Samples from various stages of the purification were subjected to SDS/PAGE and the gel stained for protein. The results in Fig. 5.7 show that there was a substantial loss of protein during the purification and that there were at least 8 protein bands observed in the CEH_{Q2} preparation. Virtually all the variation in the recovery, and purification factor stated above occurred at the second QAE-Sephadex chromatography step as the resolution on this column was not as consistent as with the first QAE-Sephadex step. This inconsistency also resulted in the protein profile on SDS/PAGE varying slightly from one preparation to another.

The CEH_{Q2} preparation was over twice as pure as the enzyme obtained by Beckett and Boyd (1977), and with the failure of both affinity chromatography steps to produce a purer enzyme preparation, the CEH_{Q2} preparation was used in further studies described in Section 6.

5.7 SUMMARY

1. CEH was purified 150-fold with approximately 25% recovery from bovine adrenocortical cytosol.
2. A non-ionic detergent, $\text{C}_{13}\text{E}_{12}$, was utilised to solubilise the enzyme from an ammonium sulphate (0-40%) precipitate. This detergent inhibits the enzyme activity and so the enzyme must be diluted to non-inhibitory detergent concentrations before it can be assayed.
3. Gradient sievorptive chromatography on QAE-Sephadex A-25 was effective in fractionating and purifying the solubilised enzyme.
4. Attempts at affinity chromatography to further purify the enzyme after gradient sievorptive chromatography were not successful.



Fig. 5.7. SDS/PAGE of samples from stages in the purification procedure. Samples from various stages of the purification were subjected to SDS/PAGE on a 7.5% gel, and stained for protein, as described in the methods section. The approximate amount of protein is given below: A, Tissue cytosol (180 μ g); B, Ammonium sulphate (0-40%) precipitate (160 μ g); C, QAE-Sephadex A-25 I (50 μ g); D, QAE-Sephadex A-25 II (CEH_{Q2}) (20 μ g); E, QAE-Sephadex A-25 II (CEH_{Q2}) (20 μ g); S, Standards (β -galactosidase (125 000), phosphorylase a (92 000), bovine serum albumin (66 000), γ -globulin (50 000; 23 500), ovalbumin (43 000)).

SECTION 6STUDIES ON PARTIALLY PURIFIED CHOLESTEROL ESTER HYDROLASEFROM BOVINE ADRENOCORTICAL CYTOSOL

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6.1 INTRODUCTION

Despite the failure of the affinity chromatography procedures to produce highly purified CEH enzyme, the CEH_{Q2} preparation represented a great improvement on previous purifications of CEH from bovine adrenocortical cytosol (Beckett and Boyd (1977)). Furthermore, although the CEH_{Q2} preparation was not pure and contained several proteins (see Fig. 5.7) it might be possible to use this as the CEH source for studies on the postulated control system (see Fig. 1.3) provided that it was free from the other components of the control system such as phosphoprotein phosphatase and cAMP-dependent protein kinase. Similarly, this preparation could be useful in the identification of the CEH enzyme protein in bovine adrenocortical cytosol. This was necessary for Krebs' criteria for phosphorylated enzymes to be fulfilled, and for final proof of a physiologically important phosphorylation of CEH in adrenal cortex. It would also be useful in the elucidation of the relationship between CEH in adrenocortical cytosol and the HSL of adipose tissue cytosol.

Although the CEH_{Q2} preparation contained several proteins, labelling studies could assist in the identification of the enzyme protein. Studies on partially purified CEH from adrenal cortex following incubation with $[\gamma\text{-}^{32}\text{P}]$ ATP have shown an increase in CEH activity with concomitant labelling of the protein fraction by ^{32}P (Trzeciak and Boyd (1974), Wallat and Kunau (1976), Beckett and Boyd (1977)). In one of these studies a subunit of Mr 41 000 was estimated for a ^{32}P -labelled protein exhibiting esterase activity (Beckett and Boyd (1977)). It had been found previously that incubation of partially purified HSL from rat adipose tissue with $[\gamma\text{-}^{32}\text{P}]$ ATP resulted in incorporation of ^{32}P radioactivity into the protein fraction with a parallel increase in enzyme activity (Huttunen, Aquino and Steinberg

(1970), Huttunen, Steinberg and Mayer (1970)). Recently it has been reported that purified HSL from chicken adipose tissue was phosphorylated with $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP, with ^{32}P incorporation into a protein with Mr 42 000 on SDS/PAGE (Berglund, Khoo et al. (1980)). Phosphorylation with $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP, of HSL purified from rat adipose tissue had indicated a molecular weight of 86 000 (Belfrage, Jergil et al. (1977)) or 84 000 (Khoo, Berglund et al. (1980), Fredrikson, Stralfors et al. (1981a)) for this enzyme. Therefore labelling the CEH enzyme with ^{32}P using $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP could assist in the identification of the enzyme protein.

Civen, Brown and Morin (1977) found that the ACTH- or cAMP-induced increase in corticoid production by isolated rat adrenal cells was blocked by minute amounts of chlorpyrifos oxone, an organophosphate insecticide, known to be an inhibitor of plasma and erythrocyte acetylcholine esterase (Civen, Lifrak and Brown (1977)). The insecticide did not affect cAMP production or affect corticoid synthesis when pregnenolone was added to the adrenal cells in vitro (Civen, Lifrak and Brown (1977)). Chlorpyrifos oxone was found to inhibit adrenal cytosolic CEH and triacylglycerol lipase (Pittman and Steinberg (1977)). It was also found that di-isopropylfluorophosphate (DFP) inhibited rat adipose tissue HSL (Belfrage, Jergil et al. (1977)) and phenylmethylsulphonylfluoride (PMSF) inhibited HSL in chicken adipose tissue (Berglund, Khoo and Steinberg (1979)) and CEH in bovine adrenal cortex (Yeaman, Cook and Lee (1980)). Furthermore, incubation of purified HSL from chicken adipose tissue with ^{14}C -labelled PMSF resulted in the labelling of a protein with Mr 42 000 on SDS/PAGE (Berglund, Khoo and Steinberg (1979)). Therefore incubation of CEH with radioactively labelled inhibitors such as these, may also assist in the identification of the enzyme protein.

As the elucidation of the control system regulating CEH activity in adrenocortical cytosol was a long term aim of this project, some experiments were performed to evaluate the potential of the CEH_{Q_2} preparation in the study of this control system. Further experiments were performed to investigate, identify and characterise, with respect to molecular weight, the CEH enzyme protein in bovine adrenocortical cytosol using the CEH_{Q_2} preparation and labelling with $[\gamma\text{-}^{32}\text{P}]$ ATP and $[1,3\text{-}^3\text{H}]$ DFP.

6.2 ENZYME ACTIVITIES IN THE CEH_{Q_2} PREPARATION - THE PARTIALLY PURIFIED CEH

The activities of the three main enzymes in the postulated CEH control system in bovine adrenocortical cytosol were determined in the CEH_{Q_2} preparation.

6.2.1 The basal CEH activity

The basal CEH activity in the CEH_{Q_2} preparation, measured after dilution (1 in 80) to reduce the detergent concentration, was found to vary considerably when apparently identical preparations were studied over a 9 month period. The average activity was 51 ± 20 (S.D.) nmol oleic acid produced $\text{min}^{-1} \cdot \text{mg protein}^{-1}$. There were two reasons for the variation observed. Firstly the figures reflect a variation in basal CEH activity in the cytosol, observed during this project. During the winter months (November-February) the basal CEH activity in the cytosol was found to be twice as high as that observed in summer (April-June). The average CEH activities in the CEH_{Q_2} preparation during these periods were 70 ± 16 (S.D.) and 37 ± 7 (S.D.) $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively. A detailed study of the possible cause of this seasonal variation in cytosolic CEH activity was not carried out during this project. Therefore statistical analysis was not possible and so the phenomenon remains simply an observation at the present time. The physiological

significance of such variation could only be speculated, but it was possible that, in response to long-term cold stress during winter, the circulating ACTH concentration was greater resulting in a trophic effect such as induction of CEH enzyme protein, rather than an effect on the phosphorylation state of the enzyme.

The second reason for the variation in the basal CEH activity in the CEH_{Q2} preparation was due to the inconsistency of the second QAE-Sephadex A-25 chromatography step. This gave not only a variation in the specific activity of the enzyme, but also a variation in the purification factor and protein profile observed by SDS/PAGE (see Section 5.6).

However, the specific activity was considerably higher than that reported in the purification of Beckett and Boyd (1977), where a 57-fold purified enzyme had a specific activity of 800 pmol.min⁻¹.mg⁻¹, but it was less than the specific activity of 300 nmol.min⁻¹.mg⁻¹ claimed by Naghshineh, Treadwell et al. (1978) for their enzyme preparation.

Both of these studies used substrate dissolved in acetone and thin-layer chromatography to assay the release of [4-¹⁴C] cholesterol from [4-¹⁴C] cholesterol oleate, and hence the activities cannot be compared directly to the specific activity observed in this study.

6.2.3 Phosphoprotein phosphatase activity

The CEH_{Q2} preparation was concentrated by pressure ultrafiltration (see Section 5.4.1) and dialysed against 10 mM potassium phosphate buffer pH 7.4 containing 30% (v/v) glycerol, 0.2 mM DTT and 0.2% C₁₃E₁₂. Phosphoprotein phosphatase activity was determined using ³²P-labelled phosphohistone as substrate. An increased amount of radiolabel appearing in the TCA-supernatant with a concomitant decrease in the radiolabel in the protein pellet indicated phosphoprotein phosphatase activity.

Table 6.1 shows that none of the incubations resulted in an increase in ^{32}P released from the protein, and the slight differences were not significant ($p>0.1$). The results for the protein pellet are more variable due to procedural errors that occur during the washing. However the differences observed were not significant ($p>0.1$). These results show that there was no phosphoprotein phosphatase activity in the CEH_{Q2} preparation. The CEH_{Q2} preparation was diluted to the same degree as for the CEH activity assays (1 in 80) and so phosphoprotein phosphatase activity was not observed at detergent concentrations at which the CEH was not inhibited. The concentrations of Mg^{2+} and Mn^{2+} chosen were known to stimulate phosphoprotein phosphatase activity in bovine adrenal cortex (Beckett and Boyd (1977), see Fig. 7.4 this thesis), but they had no effect in this experiment.

6.2.3 Protein kinase activity

The protein kinase activity in the concentrated CEH_{Q2} preparation was determined using $[\gamma\text{-}^{32}\text{P}]$ ATP and histone as described in the methods section. Increased ^{32}P incorporation into the protein pellet indicated protein kinase activity.

Table 6.2 shows that, in certain cases, the CEH_{Q2} preparation stimulated the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]$ ATP into the TCA precipitated protein compared to the control assays without CEH_{Q2} preparation. Of the three protein kinase activities assayed, cAMP-dependent, cAMP-independent and Ca^{2+} -dependent, only the first two activities were detected, although even those activities were low. There was variation in the different control incubations and thus the significance of the result obtained in the Ca^{2+} -dependent protein kinase assay (8.1 pmol) was difficult to estimate. This figure was less than that obtained for the CEH_{Q2} preparation alone, hence the presence of Ca^{2+} appeared to inhibit the cAMP-independent protein kinase activity. However there was

Additions to ^{32}P - labelled phosphohistone	^{32}P Content (pmol)	
	Supernatant	Pellet
None	28.7 \pm 1.9	676.4 \pm 28.9
CEH _{Q2} preparation	25.3 \pm 1.3	672.0 \pm 26.6
10 mM Mg ²⁺	27.3 \pm 1.2	719.7 \pm 35.9
CEH _{Q2} preparation + 10 mM Mg ²⁺	27.9 \pm 0.6	728.3 \pm 14.3
1 mM Mn ²⁺	25.9 \pm 0.6	710.1 \pm 36.8
CEH _{Q2} preparation + 1 mM Mn ²⁺	27.0 \pm 0.2	709.9 \pm 10.4

Table 6.1. Phosphoprotein phosphatase activity in the CEH_{Q2} preparation. 50 μl ^{32}P -labelled phosphohistone (1 mgml^{-1} , 4 $\mu\text{Ci.ml}^{-1}$) prepared as described in the methods section, was incubated for 30 min at 37°C with the additions stated above in a total volume of 400 μl . The phosphoprotein phosphatase activity was determined as described in the methods section. CEH_{Q2} preparation (0.4 mgml^{-1}) in 10 mM potassium phosphate buffer pH 7.4 containing 30% (v/v) glycerol, 0.2 mM DTT and 0.2% C₁₃E₁₂ was diluted 1 in 80 in the assay. Mg²⁺ and Mn²⁺ were the chloride salts in distilled water. The results are expressed as means \pm S.E.M. of triplicate assays.

Additions to $\left[\gamma\text{-}^{32}\text{P} \right] \text{ATP}$	^{32}P incorporated into histone (pmol)
Buffer	6.8 ± 1.3
Assay buffer	7.5 ± 2.4
Assay buffer + CEH_{Q2} preparation	14.6 ± 1.5
Assay buffer + 100 μM cAMP	5.7 ± 0.4
Assay buffer + CEH_{Q2} preparation + 100 μM cAMP	19.8 ± 1.0
Assay buffer + 2.0 mM Ca^{2+}	4.4 ± 0.7
Assay buffer + CEH_{Q2} preparation + 2.0 mM Ca^{2+}	8.1 ± 1.1

Table 6.2. Protein kinase activities in the CEH_{Q2} preparation. The incubations contained 0.1 mM $\left[\gamma\text{-}^{32}\text{P} \right] \text{ATP}$ ($352 \text{ cpm.pmol}^{-1}$) prepared as described in the methods section. The assay buffer contained 2-glycerophosphate (50 mM), calf thymus histone (240 μg), sodium fluoride (20 mM), theophylline (2 mM), magnesium acetate (3.5 mM) and EGTA (0.3 mM), adjusted to pH 6.0. The figures indicate the final concentrations in the assay. CEH_{Q2} preparation was as described in the legend to Table 6.1 and was diluted 1 in 40 in these assays. cAMP and Ca^{2+} (calcium chloride) were in 20 mM Tris-HCl buffer pH 7.4 at 37°C and all incubations were made up to 100 μl with this buffer (Buffer). Incubation was for 10 min at 30°C and ^{32}P incorporated into histone was determined as described in the methods section. Results are expressed as means \pm S.E.M. of triplicate assays.

a small, but significant stimulation of ^{32}P incorporation into histone by the CEH_{Q2} preparation without cAMP ($p < 0.02$) and with cAMP ($p < 0.05$). Taking an average control value of 6.6 pmol (7.5 and 5.7) from these figures the specific activities of cAMP-independent and cAMP-dependent protein kinases' were 27 and 16 pmol ^{32}P incorporated. $\text{min}^{-1}.\text{mg CEH}_{\text{Q2}}$ protein $^{-1}$, respectively. This was low in comparison to the specific activity of CEH in the CEH_{Q2} preparation (see Section 6.2.1). However it must be noted that the dilution was only 40-fold in this experiment, consequently detergent inhibition may be reflected in these activities.

6.2.4 Modulation of CEH activity

The previous experiments had shown that there was residual cAMP-dependent and independent protein kinase activities, but no phosphoprotein phosphatase activity in the CEH_{Q2} preparation when measured against commonly used substrates. However as it was the postulated control system for CEH that was under investigation, a more specific assay for protein kinase and phosphoprotein phosphatase activities would be to use the CEH_{Q2} preparation as the substrate. As it was known that this enzyme preparation contained many proteins (see Section 5.6) it was thought that the preparation was not sufficiently pure to give results for either assay if ^{32}P incorporation and release were used in the kinase and phosphatase assays. Therefore it was decided to investigate the modulation of CEH activity in the CEH_{Q2} preparation, to determine if there was a specific phosphoprotein phosphatase for CEH or if the cAMP-dependent protein kinase activity observed previously could activate CEH. The CEH_{Q2} preparation was preincubated with Mg^{2+} , $\text{Mg}^{2+}/\text{ATP}$ or $\text{Mg}^{2+}/\text{ATP}$ plus cAMP, cGMP or Ca^{2+} before the addition of cholesterol $[1-^{14}\text{C}]$ oleate substrate emulsion and assay of the CEH activity.

The experiment was performed as described in the legend to Fig. 6.1 because it was discovered that preincubation of small amounts of CEH_{Q2} protein at 40-fold dilution and 37°C led to inactivation of the enzyme. This did not occur during normal CEH assays due to the presence of the substrate which protects the enzyme. The presence of 0.02% bovine serum albumin (see legend to Fig. 5.3) may also prevent inactivation but this was omitted from the preincubations due to other effects, such as binding, that it may exert. A concentrated CEH_{Q2} preparation was not used as the concentration procedure often led to substantial losses of CEH activity.

Fig. 6.1 shows that preincubation of CEH_{Q2} preparation with Mg²⁺ did not significantly alter the CEH activity. Similarly preincubation of the CEH_{Q2} preparation with Mg²⁺/ATP or Mg²⁺/ATP plus cGMP or Ca²⁺ did not significantly affect the CEH activity. However, preincubation with Mg²⁺/ATP in the presence of cAMP led to a significant increase in the specific activity of CEH in the CEH_{Q2} preparation ($p < 0.005$). Inclusion of exogenous cAMP-dependent protein kinase (bovine heart) with the cAMP and Mg²⁺/ATP led to a further significant increase in the specific activity of CEH. These results show that there was no specific phosphoprotein phosphatase in the CEH_{Q2} preparation capable of deactivating CEH under conditions known to stimulate the deactivation of CEH in crude preparations (Trzeciak and Boyd (1974), Wallat and Kunau (1976), Beckett and Boyd (1977)). Preincubation with Mg²⁺/ATP showed that there was no modulation of CEH activity by cAMP-independent protein kinase and that additions of cGMP and Ca²⁺ did not alter this. However, the residual cAMP-dependent protein kinase, previously observed in the CEH_{Q2} preparation, was able to activate CEH, giving a 32% increase in specific activity. Addition of exogenous cAMP-dependent protein kinase with the cAMP led to a 55% increase in the specific activity of CEH.

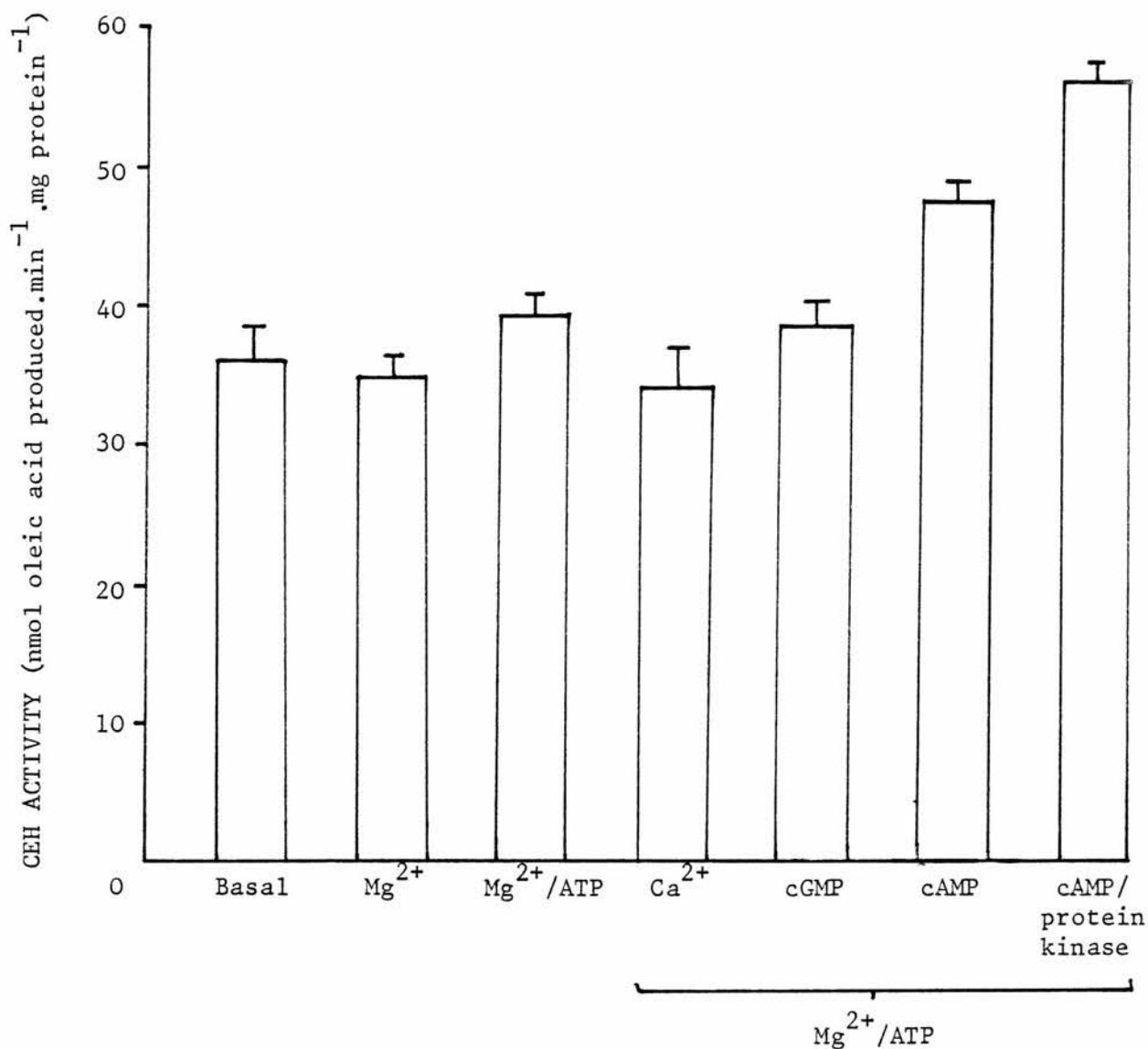


Fig. 6.1. Modulation of CEH activity in the CEH_{Q2} preparation.

The CEH_{Q2} preparation was dialysed against 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 0.2 mM DTT, 50% (v/v) glycerol and 0.1% (w/v)

C₁₃E₁₂. 1 ml samples of the preparation (0.036 mgml⁻¹) were preincubated for 10 min at 37°C without any additions, with 10 mM Mg²⁺, 10 mM ATP/Mg²⁺, 10 mM ATP/Mg²⁺ with either 2 mM Ca²⁺, 25 µM cGMP, 20 µM cAMP or 20 µM cAMP/cAMP-dependent protein kinase (20 µg), all in 20 mM Tris-HCl buffer pH 7.4 at 37°C, in a total volume of 2.1 ml. 10 µl samples from each incubation were assayed for CEH activity after dilution with 0.02% bovine serum albumin in 20 mM potassium phosphate buffer pH 7.4 (see Section 5.3). The results are means \pm S.E.M. of triplicate assays.

Again detergent inhibition may have played a role in the results observed. It was noted previously that the experiment was performed in a particular manner to avoid detergent-induced inactivation of the CEH activity. However it was not known if the detergent inhibited phosphoprotein phosphatase or protein kinase activities. In this experiment the detergent concentration in the preincubate was just under 0.05% which was 50 times higher than the non-inhibitory concentration for CEH activity. Despite this the results were in good agreement with those obtained in Sections 6.2.2 and 6.2.3, in which the detergent concentration was more dilute. Therefore the results suggested that the detergent does not have a serious effect on the activity of cAMP-dependent protein kinase.

Overall the results indicated that at least one further purification step must be included to remove the residual cAMP-dependent protein kinase activity before this preparation would be suitable for detailed studies on the postulated control mechanism. It would also be necessary to investigate the levels of heat-stable inhibitors in this preparation after the protein kinase had been removed, as these were not determined in this study. However, detergent inhibition of CEH and possibly of the other activities suggested that this purification procedure may not yield a suitable CEH enzyme preparation for the study of a complex system, with several different interacting components, such as the postulated control system (Fig. 1.3).

6.2.5 Triacylglycerol hydrolase (lipase) activity

It was not possible to measure the triacylglycerol hydrolase activity in the CEH_{Q2} preparation due to detergent inhibition coupled to the inherent lower activity of this enzyme in adrenal cortex. Gorban (1980) found that CEH activity was 1.5-3.5 fold greater than the triacylglycerol hydrolase activity in both rat adrenal cytosol and

bovine adrenocortical cytosol. This was in agreement with previous work which showed the activity of CEH in rat adrenal cytosol was some 2.5-3.0 fold higher (Gorban and Boyd (1977)) or 2.0 fold higher (Pittman and Steinberg (1977)) than the triacylglycerol lipase.

When fractions from the first QAE-Sephadex A-25 chromatography step were assayed for triacylglycerol lipase activity using the same dilution as for the CEH assay, there was a small peak of activity coeluting with the CEH peak. The activity was four fold lower than the CEH activity. When fractions from the second QAE-Sephadex A-25 chromatography step were assayed for triacylglycerol lipase activity using a 1 in 80 dilution which was twice the dilution used in the CEH assay, no activity was detected. The CEH_{Q2} preparation was also assayed at an 80-fold dilution using glycerol tri- $[1-^{14}\text{C}]$ oleate substrate emulsion with a 3-fold greater specific radioactivity for 1 h at 37°C , but this did not overcome the detergent inhibition. It was not practical to dilute the enzyme further as the amount of enzyme protein in the assay was already extremely low ($<1\ \mu\text{g}$). The peak fraction for CEH activity at the second QAE-Sephadex A-25 step was only 2.5-4.5 times the background count (60-70 cpm) after dilution. Therefore due to the dual problems of detergent inhibition and inherent lower activity the triacylglycerol lipase activity in the CEH_{Q2} preparation was unable to be measured with glycerol tri- $[1-^{14}\text{C}]$ oleate substrate emulsion.

Workers investigating the HSL of adipose tissue do not always use glycerol tri- $[1-^{14}\text{C}]$ oleate as the substrate for the enzyme. One reason for this is that the HSL enzyme also has diacylglycerol lipase activity and therefore further hydrolysis of the substrate can occur after the cleavage of the first fatty acid (Khoo and Steinberg (1974), Khoo, Steinberg et al. (1978), Steinberg (1976)). There is also a highly

active monoacylglycerol lipase in adipose tissue cytosol (Tornqvist and Belfrage (1976), Berglund, Khoo et al. (1980)). This can therefore add to the activity observed by hydrolysis of monooleoylglycerol produced by the action of HSL.

In the purification of HSL from chicken adipose tissue using Triton X-100 solubilisation, the activity of HSL was assayed towards diacylglycerol, as the enzyme was 20 times more active against that substrate than the trioleoylglycerol substrate (Berglund, Khoo et al. (1980)). Similarly, in the purification of HSL from rat adipose tissue using $C_{13}E_{12}$ solubilisation, the HSL activity was assayed using a synthetic substrate, 1(3)-mono- $[^3H]$ oleoyl-2-O-ole ylglycerol (Fredrikson, Stralfors et al. (1981a), Tornqvist, Bjorgel et al. (1978)). The enzyme was 10 times more active against this monoether analogue of diacylglycerol than with triacylglycerol, and hydrolysis did not provide a substrate for the monoacylglycerol lipase.

The activities of both CEH and triacylglycerol lipase (HSL) are 3-fold higher in adipose tissue than adrenal cortex (Gorban and Boyd (1977)). Therefore in the purification of HSL from rat adipose tissue, it was possible to detect HSL activity in the purified preparation using trioleoylglycerol substrate, despite $C_{13}E_{12}$ inhibition (Fredrikson, Stralfors et al. (1981a)). However the activities of diacylglycerol lipase and monoacylglycerol lipase in adrenocortical cytosol, and their relationships to the CEH in the tissue have not been studied.

6.2.6 Inhibition of CEH activity by di-isopropylfluorophosphate (DFP)

CEH and HSL have been shown to be inactivated by agents that are known to covalently bind to essential or active-site serine residues (see Sigman and Mooser (1975)). Both CEH and triacylglycerol lipase activities were inhibited by chlorpyrifos oxone in the 10^{-8} - 10^{-6} M range in rat adrenal (Pittman and Steinberg (1977)) and bovine adrenal

cortex (Gorban (1980)). The CEH activity may be more sensitive to the inhibition than the triacylglycerol lipase activity in rat adrenal (Pittman and Steinberg (1977)). Phenyl methyl sulphonylfluoride (PMSF) in the micromolar range had been shown to inhibit HSL in chicken adipose tissue (Berglund, Khoo and Steinberg (1979)) and CEH in bovine adrenal cortex (Yeaman, Cook and Lee (1980)). In bovine adrenal cortex the CEH activity was more sensitive to PMSF treatment than the triacylglycerol lipase activity (Yeaman, Cook and Lee (1980)). It had been reported that DFP at 50 μM could inhibit partially purified HSL from rat adipose tissue by 50% (Belfrage, Jergil et al. (1977)).

It was decided to examine the inhibition of CEH in the CEH_{Q2} preparation by a single dose of DFP. More recent work had reported a 50% inhibition of both HSL and CEH in rat adipose tissue at a DFP concentration of 10 μM (Fredrikson, Stralfors et al. (1981a)), consequently a concentration of twice this was used. A 1 ml sample of CEH_{Q2} preparation was incubated with 20 μM DFP (25 μl in propyleneglycol) for 30 min at 4°C. After incubation the sample was extensively dialysed and the CEH activity measured and compared to a control sample (1 ml CEH_{Q2} preparation + 25 μl propylene glycol), that was subjected to the same procedure without DFP.

Table 6.3 shows that 20 μM DFP inhibited the CEH activity in the CEH_{Q2} preparation by 90%. This was in agreement with the previous work using other agents, where it was found that chlorpyrifos oxone at 1 μM inhibited rat adrenal CEH by 98% (Pittman and Steinberg (1977)) and bovine adrenocortical CEH by 92% (Gorban (1980)). Similarly, PMSF at 10 μM inhibited bovine adrenocortical CEH by 80% (Yeaman, Cook and Lee (1980)). The site or sites at which these reagents interact with CEH or HSL are not known, although it was found that some stimulation of chlorpyrifos oxone-inhibited CEH was possible by incubation for 10 min

Sample	CEH Activity (nmol oleic acid produced.min ⁻¹ .mg protein ⁻¹)
Control	43.4 \pm 5.1
20 μ M DFP-treatment	4.2 \pm 1.1

Table 6.3. Inhibition of CEH activity by DFP. A sample of 1 ml CEH_{Q2} preparation in 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 0.2 mM DTT, 0.2 mM EDTA, 15 mM NaCl, 50% (v/v) glycerol and 0.1% C₁₃E₁₂ was incubated for 30 min at 4°C with 25 μ l [1,3-³H] DFP (in propylene glycol, final DFP concentration 20 μ M, 125 μ Ci). A control sample of 1 ml CEH_{Q2} preparation was incubated with 25 μ l propylene glycol. After incubation both samples were extensively dialysed against the above buffer at 4°C. Samples were diluted 80 fold for the assay of CEH activity as described in the legend to Table 5.1. Results are expressed as means \pm S.E.M. of triplicate assays.

with ATP/Mg²⁺ and cAMP (Gorban (1980)). Furthermore both cAMP-activated and Mg²⁺-deactivated CEH from bovine adrenal cortex were inhibited to the same extent by chlorpyrifos oxone (Gorban (1980)) suggesting that the phosphophorylation site was not a site of action for these inhibitors.

6.3 LABELLING OF THE CEH_{Q2} PREPARATION I - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

It was found that incubation of bovine adrenocortical cytosol with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ led to ³²P-labelling of the protein fraction with a concomitant 122% increase in CEH activity (Trzeciak and Boyd (1974)). Chromatography of the phosphorylated protein on Sephadex G-200 revealed that the ³²P radioactivity peak was associated with CEH activity. Incubation of the ³²P-labelled protein with 5 mM Mg²⁺ resulted in the loss of ³²P-labelled phosphate from the phosphorylated protein with a concomitant decrease in CEH activity. These results were supported by Wallat and Kunau (1976) where ³²P radioactivity from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was incorporated into the protein fraction of bovine adrenocortical cytosol. The protein-bound ³²P radioactivity essentially copurified with the CEH activity during ammonium sulphate precipitation or acid precipitation followed by gel permeation chromatography.

Beckett and Boyd (1977) described a purification of ³²P-phosphorylated CEH, in which a ³²P-labelled ammonium sulphate precipitate prepared from the cytosol was subjected to chromatography on Sephadex G-200 and Sepharose 4B. This procedure separated the phosphorylated CEH protein from some other phosphorylated proteins. The molecular weight of the purified CEH preparation was estimated at 173 000 by gel permeation chromatography. Electrophoresis of the phosphorylated enzyme on native gels gave two proteins, but only the one of higher electrophoretic mobility was labelled with ³²P and an esterase activity stain (1-naphthol propionate as substrate). SDS/PAGE of the same sample showed two main proteins in close proximity with the radioactivity associated more with the band of greater mobility, with an estimated molecular weight of 41 000.

Therefore there was good evidence that the activation of CEH by cAMP required the transfer of the terminal phosphate from ATP to the enzyme protein. This could be used to label the enzyme protein when $[\gamma\text{-}^{32}\text{P}]$ ATP was used in the activation reaction. However the study by Beckett and Boyd (1977) was the only one where the identification of the CEH enzyme protein was attempted. The CEH preparation in this study was only 57-fold purified with a specific activity of 0.8 nmol oleic acid produced.min⁻¹.mg protein⁻¹ and a major assumption was made in the comparison of native and SDS polyacrylamidegels of the purified material. The CEH_{Q2} preparation, purified 150-fold with a specific activity of approximately 50 nmol oleic acid produced.min⁻¹.mg protein⁻¹ was now available. Furthermore, modern slab gel techniques provide greater resolution and sensitivity than could be achieved with disc gel electrophoresis. Therefore it was decided to reinvestigate ³²P-labelling of CEH using $[\gamma\text{-}^{32}\text{P}]$ ATP as a technique for the identification of the CEH enzyme protein.

6.3.1 Analysis of ³²P-labelled proteins by autoradiography

Samples of concentrated CEH_{Q2} preparation (specific activity = 70 nmol oleic acid produced.min⁻¹.mg protein⁻¹ and 122-fold purified) were incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP, Mg²⁺ and cAMP with or without added exogenous cAMP-dependent protein kinase (type II, bovine heart), as described in the methods section. The reaction was terminated by the addition of ice-cold 25% (w/v) TCA and the precipitated protein washed with ice-cold 5% (w/v) TCA and acetone. The protein pellets were dried by vacuum desiccation and subjected to SDS/PAGE as described in the methods section. The dried gel was autoradiographed without an intensifying screen as described in the methods section.

The gel, stained for protein, and the corresponding autoradiograph are shown in Fig. 6.2. As was observed previously (Fig. 5.7),

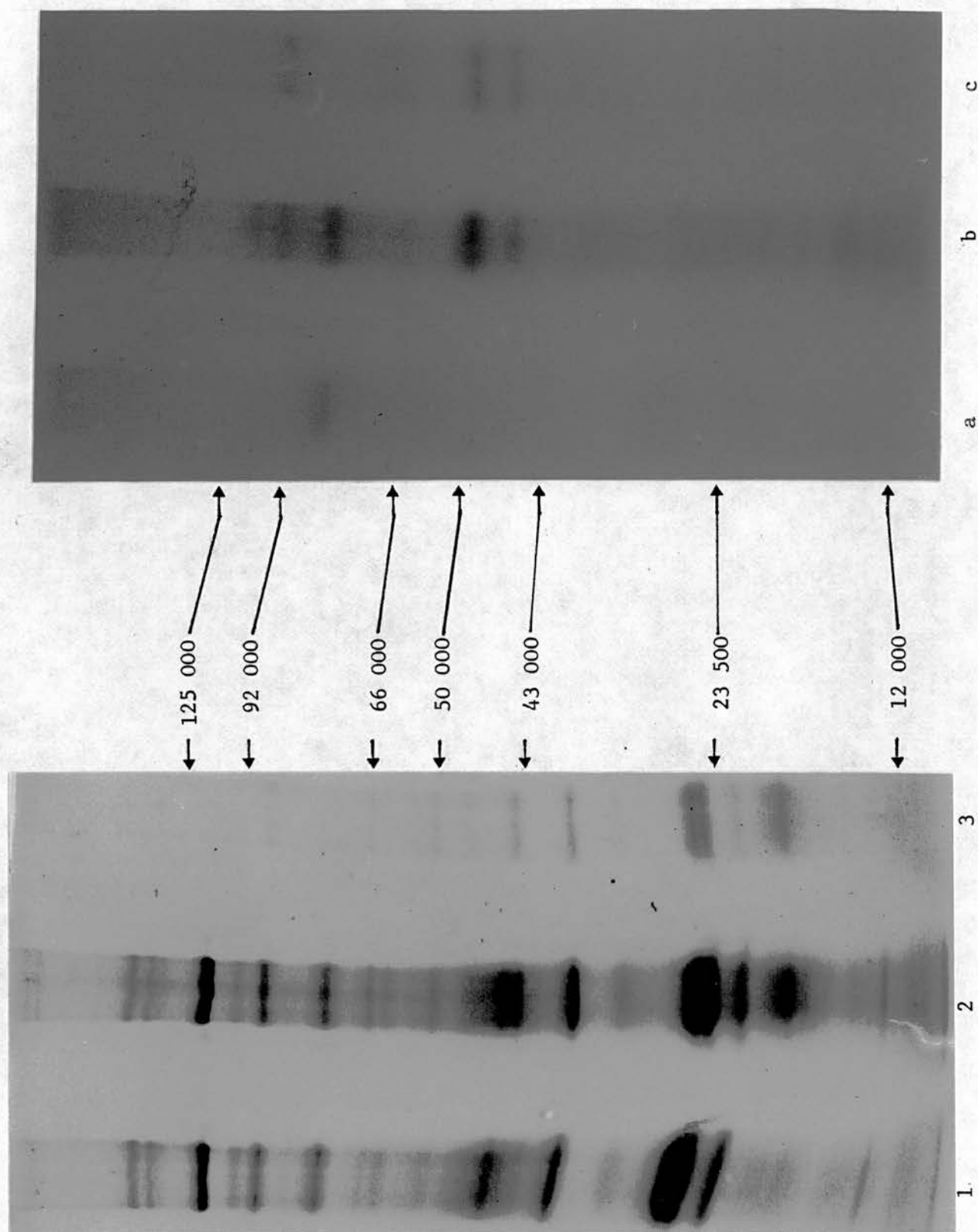


Fig. 6.2.

Legend to Fig. 6.2. ^{32}P -labelling of CEH_{Q2} preparation with $\left[\gamma\text{-}^{32}\text{P}\right]\text{ATP}$; Autoradiography. The CEH_{Q2} preparation was concentrated by pressure ultrafiltration as described in Section 5.4.1 and dialysed against 20 mM Tris-HCl buffer pH 7.0 at 37°C containing 0.2 mM DTT. Samples of this preparation (100 μg protein) were incubated for 30 min at 37°C with 0.3 mM $\left[\gamma\text{-}^{32}\text{P}\right]\text{ATP}$ (50 μCi , 400 cpm.pmol $^{-1}$), 5 mM magnesium chloride and 50 μM cAMP in 50 mM Tris-HCl buffer pH 7.4 at 37°C . The reaction was terminated by the addition of ice-cold 25% (w/v) TCA and the samples prepared for SDS/PAGE on a 6-12% exponential gradient gel as described in the methods section. The dried gel, stained for protein was subjected to autoradiography as described in the methods section. Slots 1, 2 and 3, protein stain; slots a, b and c, autoradiograph.

Slots 1,a - CEH_{Q2} preparation plus $\left[\gamma\text{-}^{32}\text{P}\right]\text{ATP}$;
 Slots 2,b - CEH_{Q2} preparation plus $\left[\gamma\text{-}^{32}\text{P}\right]\text{ATP}$ and exogenous cAMP-dependent protein kinase (20 μg , type II bovine heart);
 Slots 3,c - $\left[\gamma\text{-}^{32}\text{P}\right]\text{ATP}$ plus exogenous cAMP-dependent protein kinase (as above).

The arrows indicate the position on the gel of molecular weight standards.

there were about 8 major proteins in the CEH_{Q2} preparation including one with Mr 80 000 and at least another 8 lesser proteins observed when the gel was stained for protein (Fig. 6.2, 1&2). However autoradiography of the gel showed that only one major band of radioactivity, corresponding to a protein with Mr 82 000-84 000, was observed when the CEH_{Q2} preparation was incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP, Mg^{2+} and cAMP (Fig. 6.2a). This was due to phosphorylation by endogenous protein kinase. When exogenous cAMP-dependent protein kinase was added to the incubation, a further 5 phosphorylated bands were observed (Fig. 6.2b), but four of these were due to contaminating proteins in the cAMP-dependent protein kinase preparation that were phosphorylated, including the regulatory subunit (Fig. 6.2c). However an additional CEH_{Q2} protein with Mr 105 000 was phosphorylated while the phosphorylation of the protein with Mr 82 000-84 000 was enhanced.

It was shown in Table 6.2 that there was residual cAMP-independent protein kinase activity and cAMP-dependent protein kinase activity in the CEH_{Q2} preparation. However only the cAMP-dependent protein kinase activated CEH in the CEH_{Q2} preparation and the activation was enhanced by the addition of exogenous cAMP-dependent protein kinase (Fig. 6.1). In the experiment performed here, it was shown that only one major protein with Mr 82 000-84 000 was phosphorylated when the CEH_{Q2} preparation was incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP in the presence of cAMP. The addition of exogenous cAMP-dependent protein kinase enhanced the phosphorylation of this protein and a further protein with Mr 105 000 was also phosphorylated. Although a control incubation without cAMP was not included in this experiment it was possible that the protein with Mr 82 000-84 000 represented the CEH enzyme protein.

6.3.2 Analysis of the ^{32}P -labelled proteins by gel slicing

Samples of concentrated CEH_{Q2} preparation were incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP, Mg^{2+} and cAMP with and without added exogenous cAMP-dependent

protein kinase as described in the previous experiment. The ^{32}P -labelled proteins were prepared for and subjected to SDS/PAGE as described in the methods section. In this case the dried gel was cut into 1 mm slices using a sharp scalpel and the ^{32}P incorporation estimated as described in the methods section.

The inconsistency between different CEH_{Q2} preparations was observed in this experiment, as six proteins were phosphorylated in this preparation without exogenous cAMP-dependent protein kinase (Fig. 6.3A) compared to only one in the previous CEH_{Q2} preparation (Fig. 6.2a). The phosphorylated proteins had Mr 82 000, 75 000, 73 000, 54 000, 33 000 and 15 000. Addition of cAMP-dependent protein kinase slightly stimulated the phosphorylation of the protein with Mr 82 000, and possibly stimulated the phosphorylation of the protein with Mr 15 000, with respect to the others (Fig. 6.3B). The addition of cAMP-dependent protein kinase also resulted in the rather broad peak at 54 000 splitting into 2-3 large peaks with Mr 56 000, 54 000 and 42 000. However these peaks were due to phosphorylated proteins, such as the regulatory subunit, in the cAMP-dependent protein kinase preparation (Fig. 6.3C). Although not so conclusive as the previous experiment, this experiment supports the hypothesis that the phosphorylated protein with Mr 82 000-84 000 may be the CEH enzyme protein.

6.3.3 The time-course of the phosphorylation pattern

The time course for the activation of CEH has been well documented. Wallat and Kunau (1976) showed that the activation reached a maximum by 15-20 min and was 50% complete by 5 min. Beckett and Boyd (1977) showed that the activation and phosphorylation closely paralleled each other during the first 5 min, but the activation had reached a maximum in 10-15 min whereas the phosphorylation still showed a slight rise. However this experiment used partially purified CEH and so other proteins were also phosphorylated.

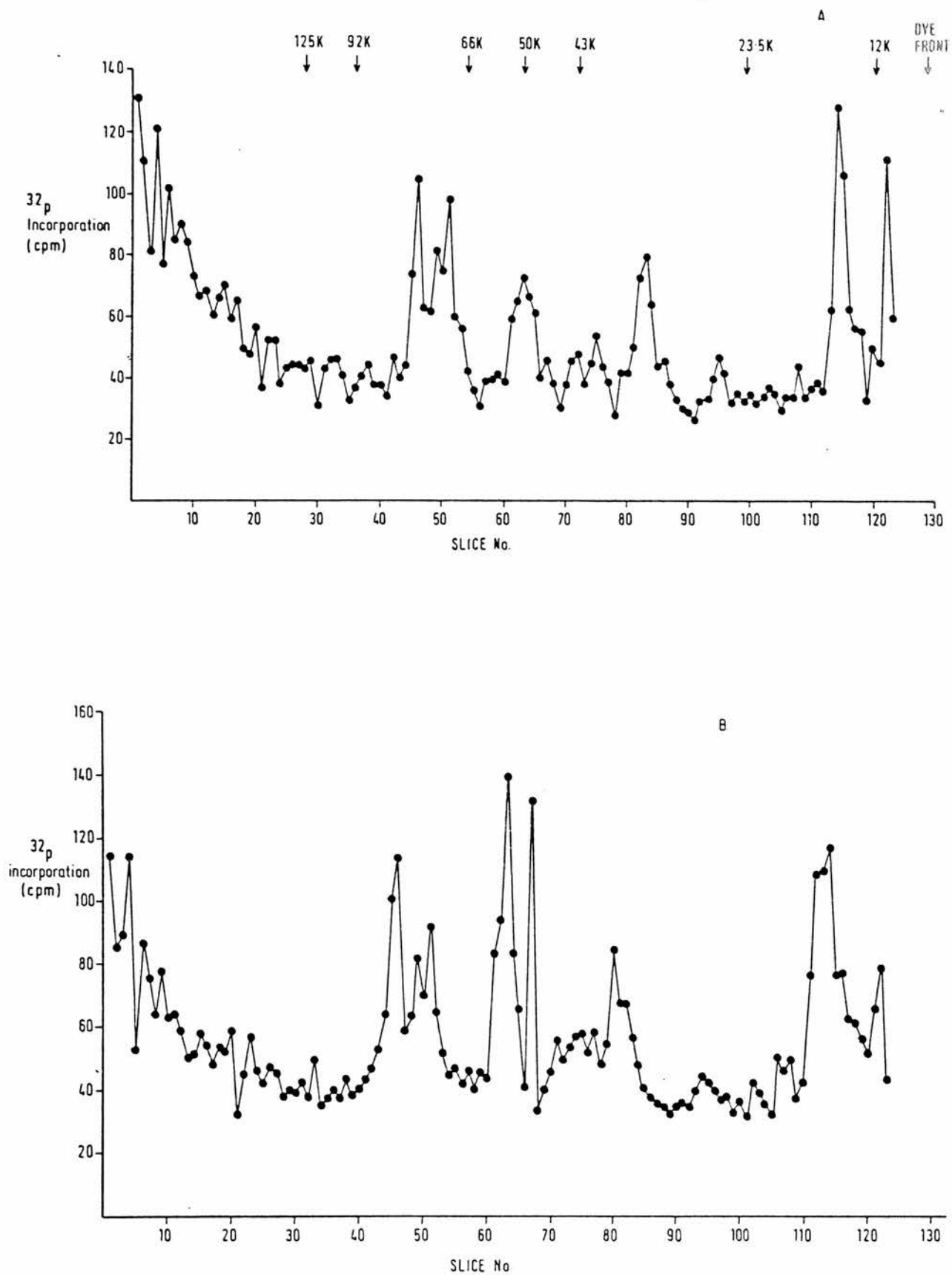


Fig. 6.3.

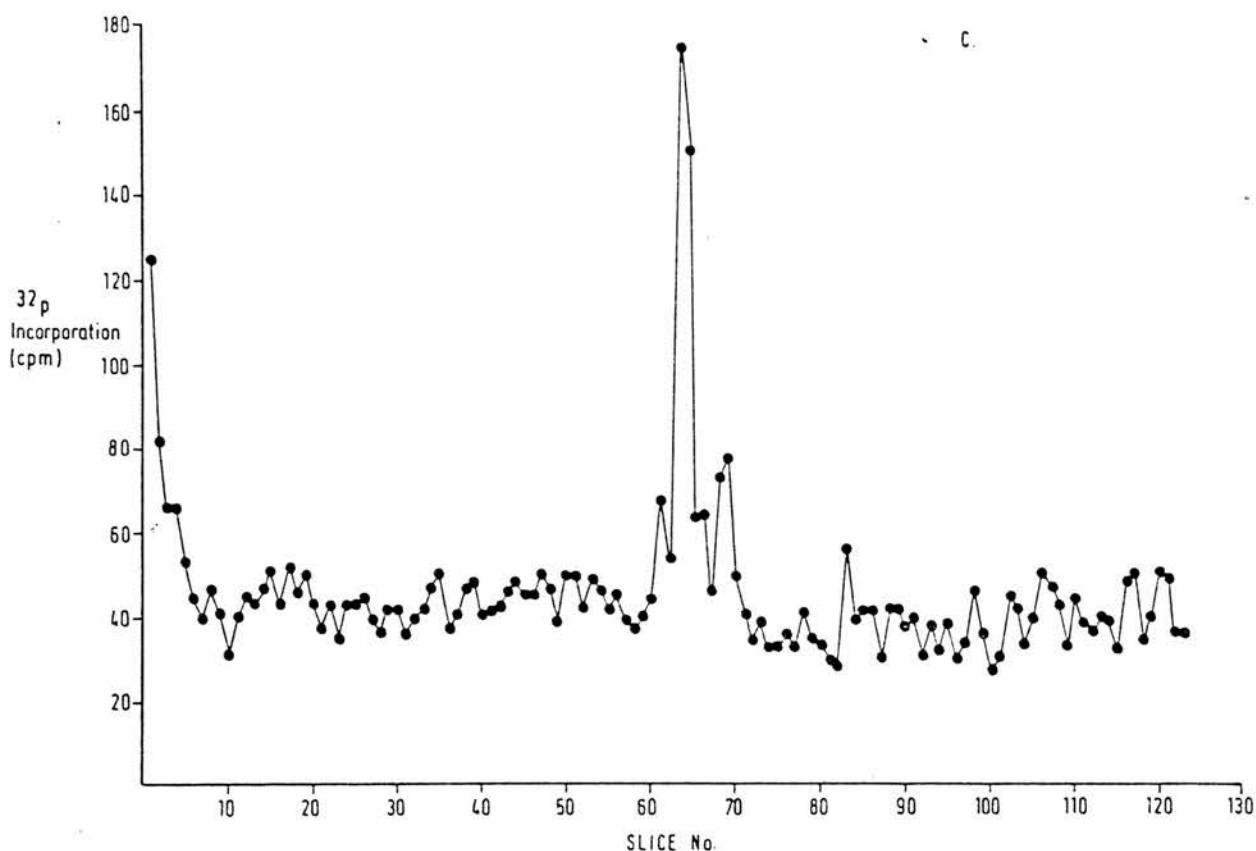


Fig. 6.3 contd. ^{32}P -labelling of $\text{CEH}_{\text{Q}2}$ preparation with $[\gamma\text{-}^{32}\text{P}] \text{ATP}$; Gel slicing. A $\text{CEH}_{\text{Q}2}$ preparation was concentrated and dialysed against 10 mM Tris-HCl buffer pH 7.0 at 37°C containing 1 mM DTT. Samples of 100 μg protein were incubated for 30 min at 37°C with 0.2 mM $[\gamma\text{-}^{32}\text{P}] \text{ATP}$ (50 μCi , 930 cpm.pmol $^{-1}$), 3 mM magnesium chloride and 30 μM cAMP in 50 mM Tris-HCl buffer pH 7.4 at 37°C . The reaction was terminated by the addition of ice-cold 25% (w/v) TCA and the samples prepared for SDS/PAGE on a 6-12% exponential gradient gel as described in the methods section. The slots on the dried gel were cut into 1 mm slices and ^{32}P incorporation estimated as described in the methods section.

A - $\text{CEH}_{\text{Q}2}$ preparation plus $[\gamma\text{-}^{32}\text{P}] \text{ATP}$;
 B - $\text{CEH}_{\text{Q}2}$ preparation plus $[\gamma\text{-}^{32}\text{P}] \text{ATP}$ and exogenous cAMP-dependent protein kinase (20 μg , type II bovine heart);
 C - $[\gamma\text{-}^{32}\text{P}] \text{ATP}$ plus exogenous cAMP-dependent protein kinase (as above).

The arrows indicate the position on the gel of molecular weight standards.

It had been shown in this study that incubation of the CEH_{Q2} preparation with cAMP, ATP and Mg²⁺ for 10 min resulted in a 32% increase in CEH activity without exogenous cAMP-dependent protein kinase and a 55% increase in the enzyme activity with exogenous cAMP-dependent protein kinase. Evidence from the previous two experiments suggests that the CEH activity may be associated with a protein with Mr 82 000-84 000. Therefore it was decided to investigate the time-course of the phosphorylation pattern observed with the CEH_{Q2} preparation.

A sample of concentrated CEH_{Q2} preparation was incubated with 10 mM Mg²⁺, 30 mM DTT, 0.12 mM [γ -³²P] ATP and the catalytic subunit of cAMP-dependent protein kinase (650 units). The reaction was initiated by the addition of the labelled ATP. Samples were withdrawn from the incubation after 1, 2, 5, 10, 15, 20, 30 and 60 min and immediately added to an equal volume of ice-cold 25% (w/v) TCA. The samples were analysed by SDS/PAGE and autoradiography (with intensifying screen) as described in the methods section.

Fig. 6.4 shows that complete phosphorylation had been achieved after 10 min incubation. In this experiment, using another CEH_{Q2} preparation and the catalytic subunit of cAMP-dependent protein kinase, the phosphorylation pattern was more complex than observed in previous experiments, with at least 12 phosphorylated proteins. However after an incubation period of 1 min, only 2 proteins were phosphorylated, with Mr 88 000 and 36 000. The lower molecular weight protein may be the catalytic subunit of cAMP-dependent protein kinase, which is now known to be autophosphorylated although its molecular weight is usually quoted at about 39 000-42 000 (Flockhart and Corbin (1982)). By 5 min several other proteins were phosphorylated including one with Mr 84 000. As with the other proteins, full phosphorylation of this protein was achieved after 10-15 min.

TIME (min)

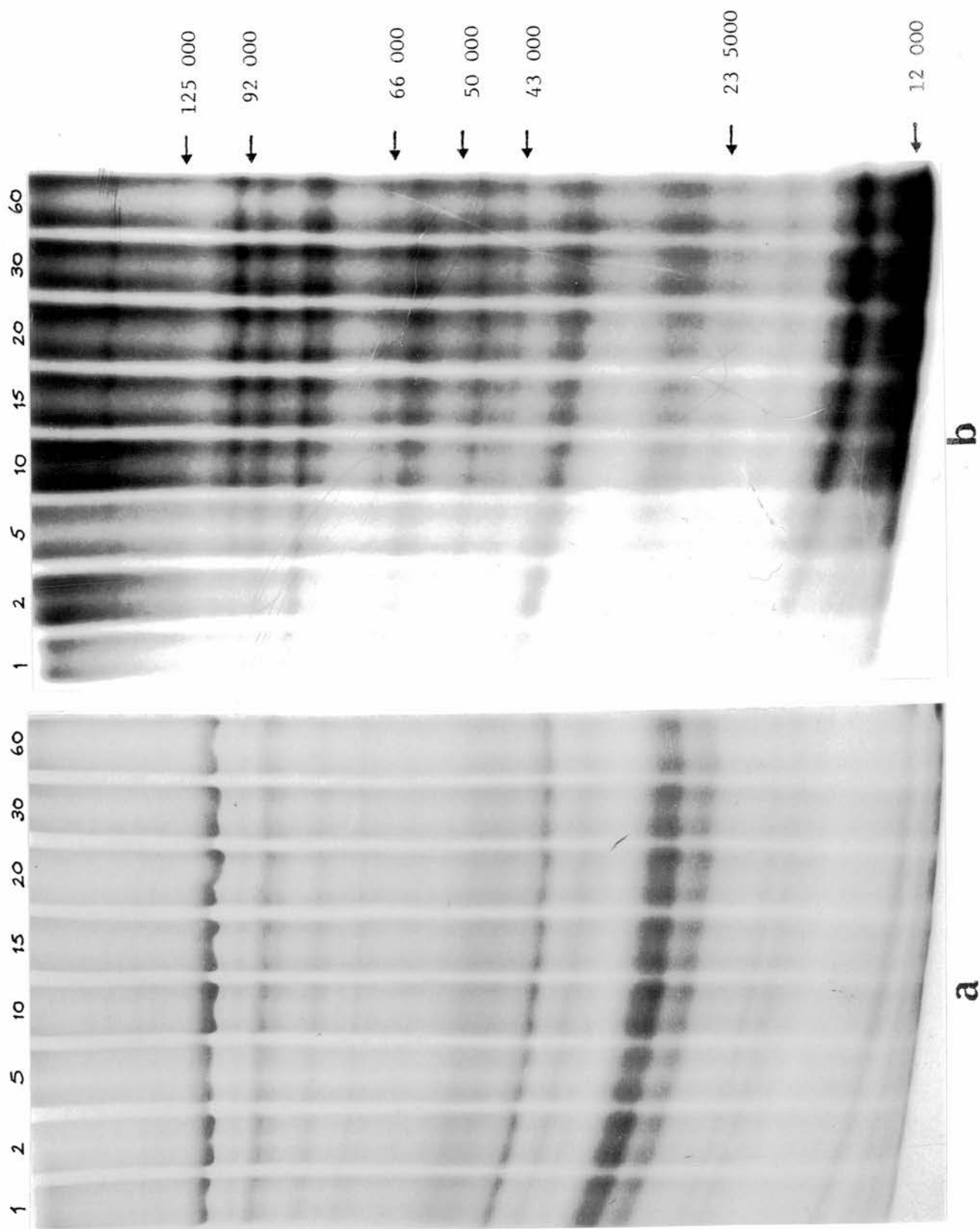


Fig. 6.4.

Legend to Fig. 6.4. Time-course of the phosphorylation pattern in the CEH_{Q2} preparation. A CEH_{Q2} preparation was concentrated by ultrafiltration and dialysed against 10 mM Tris-HCl buffer pH 7.0 at 37°C containing 0.5 mM DTT. A sample of 8 ml of the preparation (600 μg protein) was incubated at 30°C with 10 mM magnesium chloride, 30 mM DTT, 0.12 mM $[\gamma\text{-}^{32}\text{P}]$ ATP (400 μCi , 630 cpm.pmol^{-1}) and the catalytic subunit of cAMP-dependent protein kinase (650 units). The incubation was initiated by the addition of the ATP and samples of 1.2 ml were extracted at 1, 2, 5, 10, 15, 20, 30 and 60 min after initiation and immediately added to an equal volume of ice-cold 25% (w/v) TCA. The samples were subjected to SDS/PAGE on a 6-12% exponential gradient gel and autoradiography as described in the methods section.

a, protein stain; b, autoradiography.

The arrows indicate the position on the gel of molecular weight standards.

These results are in agreement with the results obtained in the previous studies showing parallel activation of CEH and phosphorylation of the protein fraction (Beckett and Boyd (1977), Naghshineh, Treadwell et al. (1978), Gorban (1980)). However, in these previous studies, the phosphorylation was measured by TCA precipitation of the protein fraction and determination of the total ^{32}P incorporation. Here, the phosphorylation was measured in a qualitative manner in order to study the phosphorylation of individual proteins. As in the previous experiment, the results shown in Fig. 6.4 were consistent with the hypothesis that the protein with Mr 82 000-84 000 was the CEH enzyme protein. The identity of the protein with Mr 88 000, which was phosphorylated very rapidly was not known, and had not been observed in the previous experiments. Further proteins with Mr's approximately 160 000, 110 000, 100 000, 70 000, 64 000, 61 000, 51 000, 45 000, 39 000, 28 000, 25 000 and 15 000 were also phosphorylated in this experiment. Some of these corresponded to those found in the previous experiment.

Overall, labelling the protein in the CEH_{Q2} preparation with ^{32}P using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was partly successful in the identification of the CEH enzyme protein. The variation in the phosphorylation pattern observed with different CEH_{Q2} preparations made identification more difficult. There was evidence, however, to suggest that CEH in bovine adrenocortical cytosol may be associated with a protein with Mr 82 000-84 000. This phosphorylated protein was found in all the cAMP-dependent phosphorylation experiments (using 5 different Q2 enzyme preparations) despite the variation observed in the phosphorylated protein pattern, and with one CEH_{Q2} preparation this was the only protein phosphorylated (Fig. 6.2). Furthermore the phosphorylation of this protein was enhanced by the addition of exogenous cAMP-dependent protein kinase (Figs. 6.2 and 6.3), was markedly phosphorylated within 5 min and fully

phosphorylated by 10-15 min after the initiation of the reaction (Fig. 6.4). However, this protein was not a major component of the CEH_{Q_2} preparation as was observed when the gels were stained for protein, although there was a relatively major protein with slightly higher electrophoretic mobility (Figs. 5.7 & 6.2). This protein was not always as prominent in other CEH_{Q_2} preparations (Fig. 6.4).

6.4 LABELLING OF THE CEH_{Q_2} PREPARATION II - $[1,3\text{-}^3\text{H}]\text{DFP}$

The results obtained from the ^{32}P -labelling experiments had suggested a possible molecular weight for the CEH enzyme protein in bovine adrenocortical cytosol. However the evidence was not conclusive and so an attempt was made to label the CEH enzyme protein in the CEH_{Q_2} preparation, more specifically, with $[1,3\text{-}^3\text{H}]\text{DFP}$. This chemical had been shown to inhibit the CEH activity in this preparation (Table 6.3).

6.4.1 Analysis of the ^3H -labelled proteins by autoradiography

A sample of concentrated CEH_{Q_2} preparation was incubated with $75\text{ }\mu\text{M}$ $[1,3\text{-}^3\text{H}]\text{DFP}$ (0.5 mCi , 6 Ci.mmol^{-1}) for 30 min at 37°C . The incubation was terminated by the addition of an equal volume of ice-cold 25% (w/v) TCA and the sample prepared for and subjected to SDS/PAGE followed by fluorography-autoradiography as described in the methods section.

Fig. 6.5 shows that 3-4 proteins were labelled by the $[1,3\text{-}^3\text{H}]\text{DFP}$, and that the major labelled protein had Mr 84 000. There was also a diffuse area of labelling with peaks corresponding to proteins with Mr 54 000 and 50 000, and possibly a third area of labelling moving just behind the dye-front corresponding to a protein with Mr 20 000. However the most densely-labelled band was due to the protein with Mr 84 000 and this corresponded with the molecular weight postulated from the results obtained in the phosphorylation experiments. There was also a diffuse area of labelling at an approximate Mr of 50 000 in

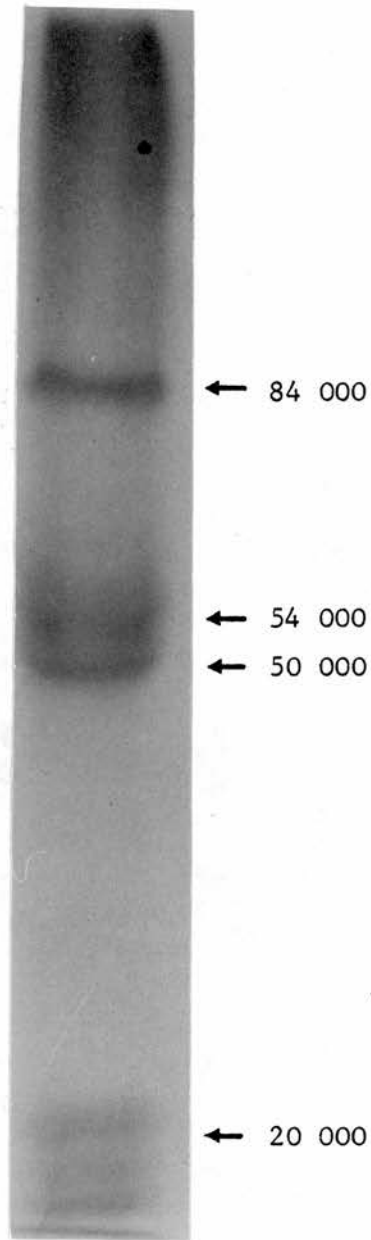


Fig. 6.5. Labelling of the CEH_{Q2} preparation with $[1,3\text{-}^3\text{H}]$ DFP; Fluorography. A CEH_{Q2} preparation was concentrated by ultrafiltration and dialysed against 10 mM Tris-HCl buffer pH 7.0 at 37°C containing 0.5 mM DTT. A sample of the preparation (150 μg protein) was incubated for 30 min at 37°C with 75 μM $[1,3\text{-}^3\text{H}]$ DFP (500 μCi , 6 Ci.mmol^{-1}). The incubation was terminated by the addition of ice-cold 25% (w/v) TCA and the sample subjected to SDS/PAGE on a 7.5% gel and fluorography as described in the methods section.

some phosphorylation experiments (Fig . 6.3A&B), but probably this was due to the regulatory subunit of type II cAMP-dependent protein kinase (Fig. 6.3C) and was not prominent in all phosphorylation experiments (Figs. 6.2 & 6.4). Similarly there was labelling of low molecular weight proteins in some phosphorylation experiments with one prominent protein with Mr 15 000, but phosphorylation of a protein with Mr 20 000 was not observed. Therefore, the protein with Mr 84 000 was the most likely candidate as the CEH enzyme protein.

6.4.2 Analysis of the ^3H -labelled proteins by gel slicing

The $[1,3-^3\text{H}]$ DFP labelling experiment was carried out as described in Section 6.4.1 except that before the gel was dried the appropriate track was excised from the gel and sectioned into 1 mm slices. The ^3H incorporation was determined by liquid scintillation counting after NCS solubilisation of the gel slices (see methods section).

Fig. 6.6 shows that the results were almost identical to those obtained from the autoradiograph, and that 4 proteins were labelled with Mr 84 000, 54 000, 50 000 and 20 000. In this experiment the proteins with similar electrophoretic mobilities were the most highly labelled, again in a rather wide band. However the protein with Mr 84 000 was again highly labelled with the low molecular weight protein labelled least.

The overall conclusion from both the $[\gamma-^{32}\text{P}]$ ATP and $[1,3-^3\text{H}]$ DFP labelling experiments was that CEH activity in bovine adrenocortical cytosol was associated with a protein of molecular weight about 84 000. It was possible that this was only a subunit of a holoenzyme, although as it was both labelled by DFP and phosphorylated it was probable that it contained catalytic and regulatory properties.

6.5 SUMMARY

1. The CEH_{Q2} preparation was used as the source of CEH activity.

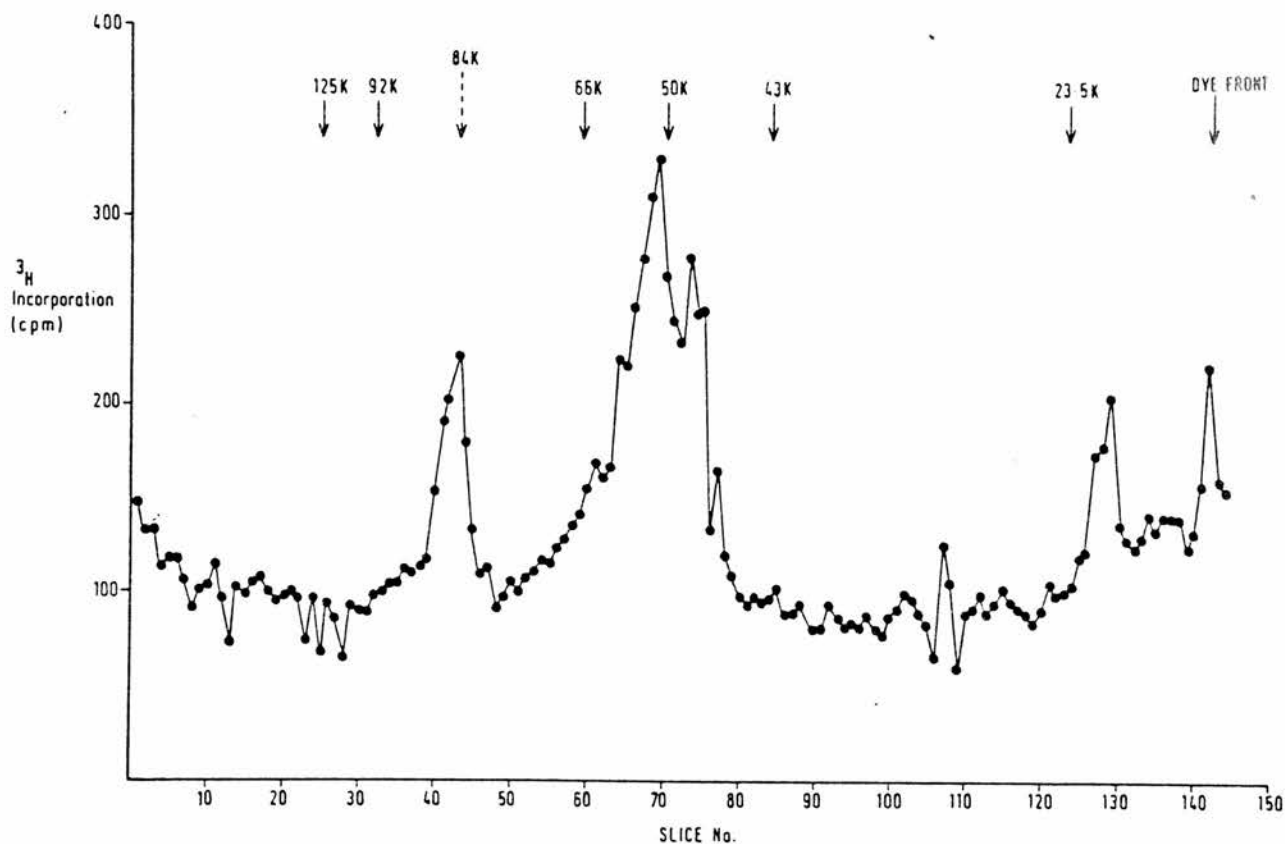


Fig. 6.6. Labelling of the $\text{CEH}_{\text{Q}2}$ preparation with $[1,3-^3\text{H}]$ DFP; Gel slicing. The experiment was performed as described in the legend to Fig. 6.5 except that before the gel was dried, this slot was cut from the gel and sectioned into 1 mm slices. ^3H incorporation was determined by liquid scintillation counting after solubilisation of the protein in the excised slices by NCS tissue solubiliser as described in the methods section. The arrows indicate the position on the gel of molecular weight standards.

2. This preparation also contained residual cAMP-independent and cAMP-dependent protein kinase activities, but was free from phosphoprotein phosphatase activity.
3. It was possible to activate CEH in this preparation by incubation with ATP/Mg²⁺ and cAMP, but incubation with Mg²⁺ alone did not alter the activity.
4. Studies using $\left[\gamma\text{-}^{32}\text{P}\right]$ ATP to label the CEH enzyme protein suggested that the activity was associated with a protein with Mr 82 000-84 000.
5. Labelling the CEH_{Q2} preparation with $\left[1,3\text{-}^3\text{H}\right]$ DFP was found to inhibit the enzyme activity, and supported the suggestion that the CEH activity was associated with a protein with Mr 84 000.

SECTION 7

INVESTIGATION OF THE EFFECT OF A PHOSPHOPROTEIN PHOSPHATASE
PURIFIED FROM BOVINE ADRENOCORTICAL CYTOSOL ON THE ACTIVITY
OF PARTIALLY PURIFIED CHOLESTEROL ESTER HYDROLASE FROM
BOVINE ADRENOCORTICAL CYTOSOL

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7.1 INTRODUCTION

CEH in the adrenal cortex cytosol is phosphorylated and activated by cAMP-dependent protein kinase (Trzeciak and Boyd (1974), Wallat and Kunau (1976)). Incubation of the activated enzyme with millimolar Mg^{2+} resulted in a time-dependent dephosphorylation and deactivation (Trzeciak and Boyd (1974)). It was also shown that incubation of the activated enzyme with bovine liver alkaline phosphatase resulted in a time-dependent deactivation of the enzyme (Beckett and Boyd (1977)). Whereas, there had been much work investigating protein kinases (reviewed Flockhart and Corbin (1982)) and their phosphorylation processes including those in the adrenal cortex, there had been relatively little work investigating the dephosphorylation processes involving phosphoprotein phosphatases (see Krebs and Beavo (1979), Glass and Krebs (1980), Hardie (1981), Cohen (1982)). However the role of phosphoprotein phosphatases in the regulation and control of metabolic pathways is now being actively investigated (see Fischer and Brautigan (1982)).

It had been shown that adrenal cortex contained an alkaline phosphatase activity (Li (1979)) and at least three distinct phosphoprotein phosphatases (Merlevede and Riley (1966), Ullman and Perlman (1975), Li (1979)). However the role of these phosphoprotein phosphatases in the regulation of cytosolic CEH activity had not been studied. Therefore, it was decided to purify a major phosphoprotein phosphatase from bovine adrenocortical cytosol and to study its effects on the activity of partially purified CEH from bovine adrenocortical cytosol.

It had been known for some time that phosphoprotein phosphatases purified from a wide range of tissues demonstrated a variety of molecular weights ranging from very high (200 000-300 000) to low (30 000-35 000). Subsequent studies resulted in the hypothesis that

the low molecular weight forms were catalytically active subunits derived from the high molecular weight forms (Brandt, Capulong and Lee (1975), Kobayashi and Kato (1977)). This catalytic subunit of about Mr 35 000 could be released from the high molecular weight forms by a variety of rather harsh treatments including 80% ethanol at room temperature (Brandt, Killilea and Lee (1974)), urea (Killilea, Mellgren et al. (1979)), freeze-thawing with 2-mercaptoethanol (Kato and Sato (1974)), limited proteolysis with trypsin (Killilea, Mellgren et al. (1979)) or Ca^{2+} -dependent protease (Mellgren, Aylward et al. (1979)) and storage at 4°C for 1-2 days (Khandelwal, Vandenheede and Krebs (1976)). However it has also been found that an Mr 260 000 phosphoprotein phosphatase could be progressively dissociated by increasing salt concentration (Khandelwal, Zinman and Ng (1980)). The catalytic subunit has a low specificity and was relatively unaffected by a variety of metabolic effectors, and has been termed the multi-functional phosphoprotein phosphatase (Krebs and Beavo (1979)).

At the time when the following experiments were performed the phosphoprotein phosphatase-free CEH_{Q2} preparation (see Section 6.2.2) was not available and so the cholate solubilised enzyme was used (see Section 3.3.4). This preparation exhibited phosphoprotein phosphatase activity in the presence of divalent cations. The endogenous phosphoprotein phosphatases would interfere in experiments with a purified phosphoprotein phosphatase and consequently it was decided to purify the multifunctional phosphoprotein phosphatase and investigate its effect on CEH activity. As this enzyme had no specific requirements for divalent cations and a low specificity it was hoped that this would eliminate interference by the endogenous phosphoprotein phosphatases present in the CEH preparation.

7.2 PURIFICATION OF THE MULTIFUNCTIONAL PHOSPHOPROTEIN PHOSPHATASE FROM BOVINE ADRENOCORTICAL CYTOSOL

A low molecular weight (Mr 35 000) phosphoprotein phosphatase of the multifunctional phosphoprotein phosphatase type had been purified from bovine adrenal cortex (Li (1979)). This enzyme was found to have low substrate specificity and was active against phosphorylase a, phosphohistone, phosphocasein and also exhibited alkaline phosphatase activity when assayed with p-nitrophenyl phosphate. Divalent cations were not required with the phosphoprotein substrates, but Mg^{2+} , Mn^{2+} or Co^{2+} and also a sulphhydryl compound were required for the alkaline phosphatase activity. However the role of the phosphoprotein phosphatase in the deactivation of CEH in the adrenal cortex was not investigated or discussed. Therefore it was decided to purify this enzyme from bovine adrenocortical cytosol and to investigate the effects of the purified phosphoprotein phosphatase on partially purified CEH from bovine adrenocortical cytosol.

The phosphoprotein phosphatase was purified essentially as described by Li (1979) with certain modifications. The enzyme source was the 105 000 x g supernatant from bovine adrenal cortex, whereas Li (1979) had used a 15 000 x g supernatant. Throughout the purification the enzyme was monitored using p-nitrophenyl phosphate and ^{32}P -labelled phosphohistone (prepared as described in the methods section) as substrates for the alkaline phosphatase and phosphoprotein phosphatase activities respectively, using the procedures detailed in the methods section. All purification steps were carried out at 4°C unless stated otherwise.

Step 1 - Tissue cytosol

The 105 000 x g supernatant was prepared from the cortex of about 40-50 bovine adrenal glands as described in the methods section.

Step 2 - Ammonium sulphate fractionation

Solid ammonium sulphate was added to the cytosol, slowly and with stirring, up to 55% saturation in the final mixture. The precipitate was collected by centrifugation and was resuspended in 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 10 mM 2-mercaptoethanol, 5 mM magnesium chloride, 1 mM EDTA and 50 mM potassium chloride (buffer A^P) and dialysed overnight against the same buffer.

Step 3 - Ethanol precipitation

The ammonium sulphate fraction had 5 volumes 95% (v/v) ethanol added to it at room temperature. The mixture was immediately centrifuged at 10 000 x g for 10 min at 20°C and the enzyme was extracted from the pellet by four successive washes with 100 ml buffer A^P. The pellet was gently homogenised with the buffer to ensure maximum recovery, and all extractions were carried out at 10°C. The combined extracts were filtered through Whatman No. 1, concentrated by precipitation with ammonium sulphate to 70% saturation and dialysed against buffer A^P overnight.

Step 4 - DEAE-cellulose chromatography

The concentrated and dialysed fraction from the previous step was loaded onto a DEAE-cellulose column (2.5 x 40 cm) equilibrated with buffer A^P. The enzyme was eluted with a linear gradient of potassium chloride (0.05-0.45 M) (Fig. 7.1a). The p-nitrophenyl phosphatase activity eluted in two peaks at 0.25 M and 0.35 M potassium chloride, whereas the phosphohistone phosphatase activity eluted in a broad peak associated with the second p-nitrophenyl phosphatase peak. At this stage, both peaks of activity were pooled as indicated in Fig. 7.1a, concentrated by precipitation with ammonium sulphate to 70% saturation and dialysed overnight against buffer A^P.

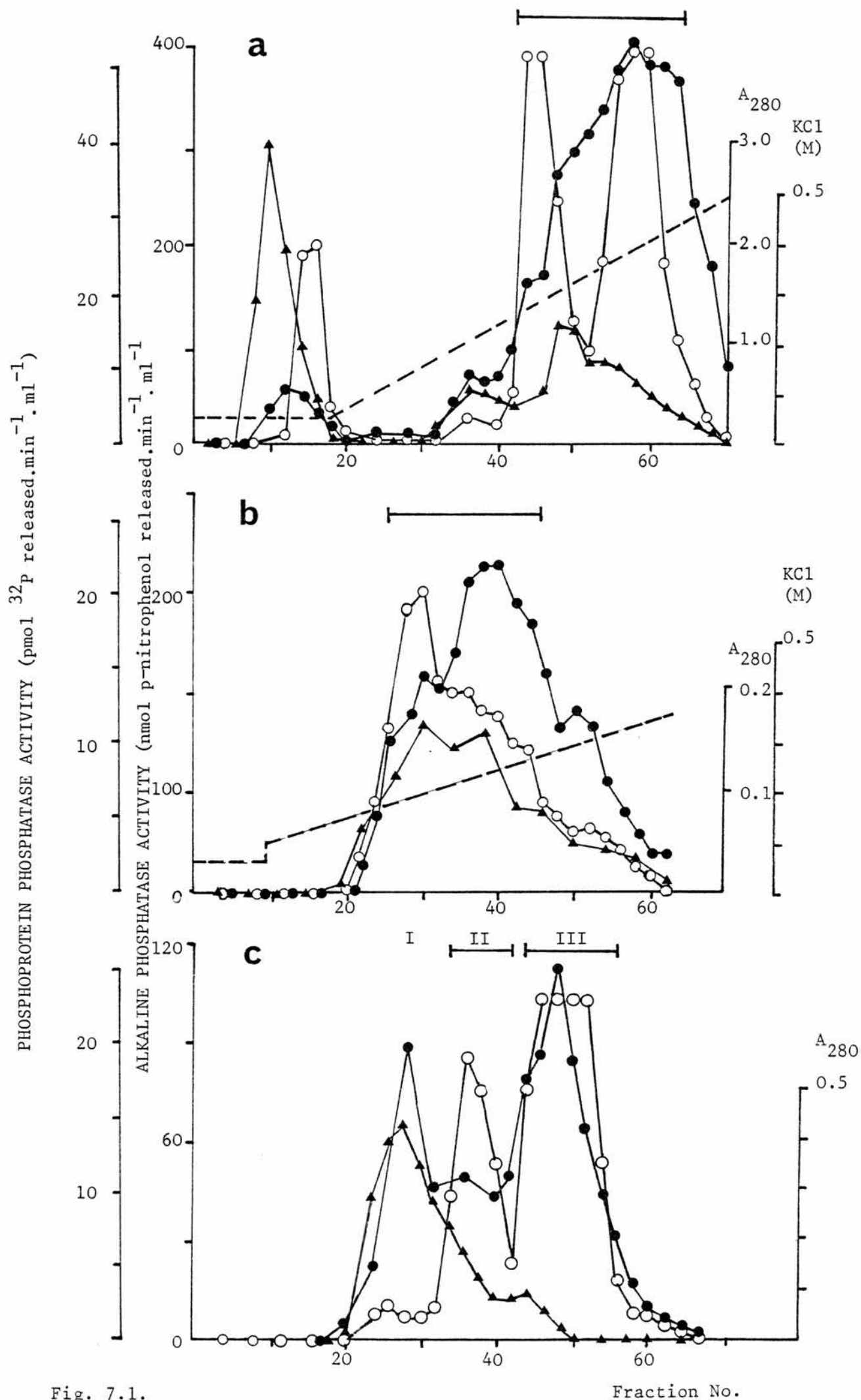


Fig. 7.1.

Legend to Fig. 7.1. Purification of the multifunctional phosphoprotein phosphatase from bovine adrenocortical cytosol.

a) DEAE-cellulose - The column (2.5 x 40 cm) was equilibrated in buffer A^P (see text) and eluted with a linear gradient of potassium chloride (0.05-0.45 M) in buffer A^P in a total volume of 500 ml. The flow rate was 80 ml.h⁻¹ and fractions of 8.2 ml were collected.

b) DEAE-Sephadex A-50 - The column (2.1 x 23 cm) was equilibrated in buffer A^P and eluted with a linear gradient of potassium chloride (0.1-0.45 M) in buffer A^P in a total volume of 600 ml. The column was operated under a pressure of 25 cm H₂O and fractions of 8.2 ml were collected.

c) Sephadex G-100 - The column (2 x 96 cm) was equilibrated and eluted with buffer C^P (see text) under a pressure of 70 cm H₂O, and fractions of 5.8 ml were collected.

³²P-labelled phosphohistone phosphatase and p-nitrophenyl phosphatase activity were measured as described in the methods section.

○ , p-Nitrophenyl phosphatase; ▲ , A₂₈₀; ● ³²P-phosphohistone phosphatase; --- , Potassium chloride concentration; └─┘ , Fractions pooled.

Step 5 - DEAE-Sephadex A-50 chromatography

The dialysed enzyme solution was applied to a DEAE-Sephadex A-50 column (2.1 x 23 cm) equilibrated with buffer A^P. The enzyme was eluted with a linear gradient of potassium chloride (0.1-0.45 M). The p-nitrophenyl phosphatase activity was eluted in a peak at 0.27 M potassium chloride with a large shoulder at about 0.33 M potassium chloride (Fig. 7.1b). As in the previous step, the phosphohistone phosphatase activity eluted as a broad peak associated mainly with the second p-nitrophenyl phosphatase peak. The fractions indicated were pooled, concentrated by pressure ultrafiltration (Amicon YM10 membrane) to about 10 ml and dialysed against 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 10 mM 2-mercaptoethanol and 50% (v/v) glycerol (buffer B^P). This resulted in a further concentration of the enzyme sample which was necessary for the next step.

Step 6 - Sephadex G-100 chromatography

The concentrated sample was subjected to gel filtration chromatography on a Sephadex G-100 column (2 x 96 cm) equilibrated with 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 10 mM 2-mercaptoethanol, 10% (v/v) glycerol and 0.1 M potassium chloride (buffer C^P). The p-nitrophenyl phosphatase activity eluted in two peaks (II and III) as shown in Fig. 7.1c. The phosphohistone phosphatase activity also eluted in two peaks (I and III) with the second peak coeluting with the second p-nitrophenyl phosphatase peak. Peak I was associated with the major protein peak and was not investigated further, but peaks II and III were pooled as indicated.

A summary of a typical purification is shown in Table 7.1. Peak III represented the greater purification of the enzyme and SDS/PAGE of samples from each stage of the procedure show a substantial purification from the crude supernatant to the peak III fraction (Fig. 7.2).

Step	Total Protein (mg)	Phosphoprotein Phosphatase			Alkaline Phosphatase		
		Specific Activity (pmol ^{32}P produced. $\text{min}^{-1} \cdot \text{mg protein}^{-1}$)	Purif. (fold)	Recov. (%)	Specific Activity (nmol p-nitrophenol produced. $\text{min}^{-1} \cdot \text{mg protein}^{-1}$)	Purif. (fold)	Recov. (%)
I. Cytosol	3654	18.2	-	100	9.7	-	100
II. Ammonium Sulphate Fraction	1716	24.8	1.4	64	14.1	1.5	68
III. 80% Ethanol Fraction	260	28.4	1.6	11	15.4	1.6	11
IV. DEAE-Cellulose	60	93.8	5.2	9	41.2	4.3	7
V. DEAE-Sephadex A-50	17.5	72.4	4.0	2	61.8	6.4	3
VI. Sephadex G-100							
peak II	1.35	203.8	11.2	1.4	238.2	24.6	6
peak III	1.65	392.3	21.6		1055	108.8	

Table 7.1. Summary of purification of the multifunctional phosphoprotein phosphatase from bovine adrenocortical cytosol. Phosphoprotein phosphatase and alkaline phosphatase activities were measured as described in the methods section. Protein concentration was measured by the method of Bradford (1976).

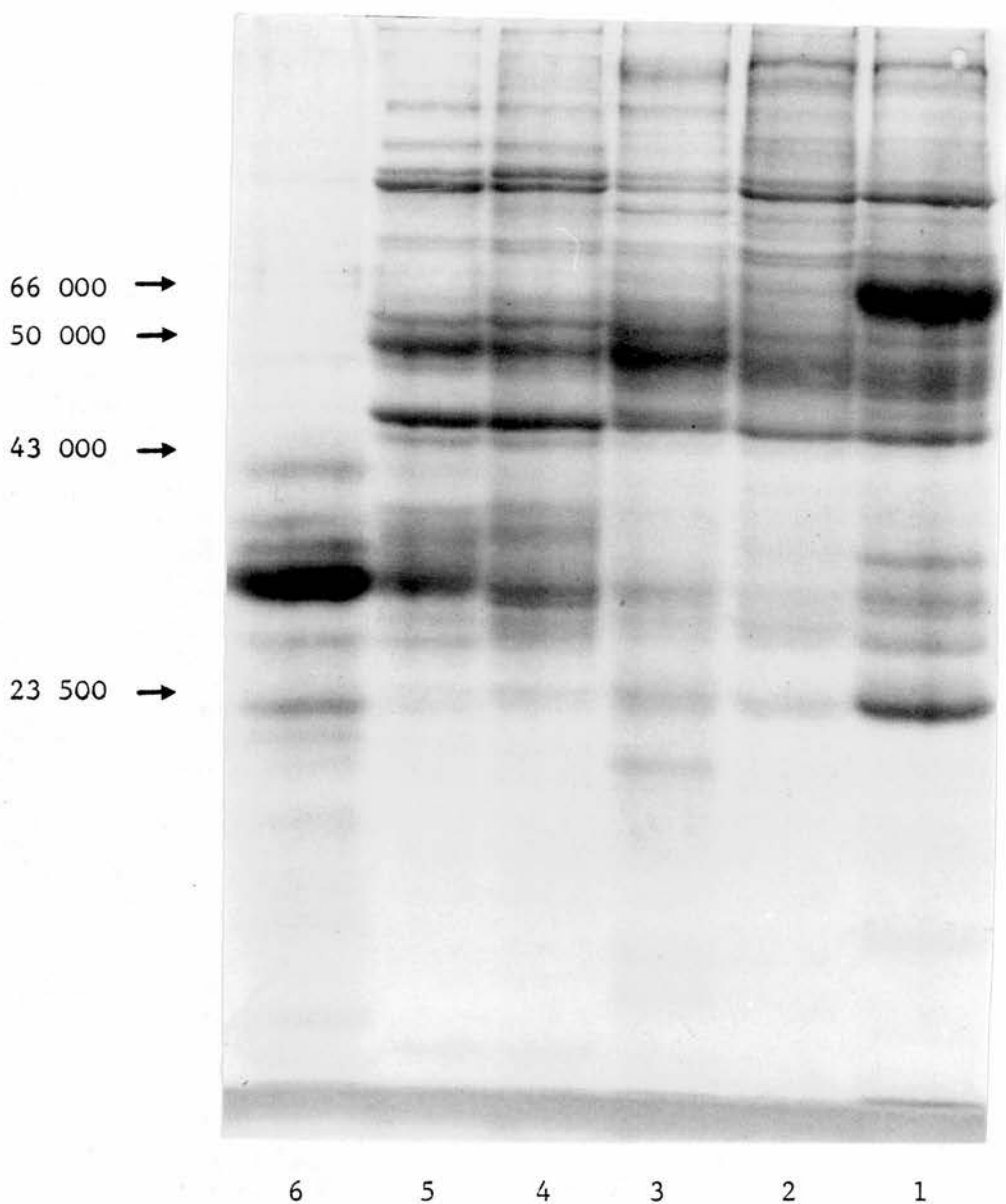


Fig. 7.2. SDS/PAGE of samples from each stage of the purification of the phosphoprotein phosphatase with Mr 35 000. Samples of approximately 100 μ g protein were subjected to SDS/PAGE on a 10% gel and stained for protein as described in the methods section. The arrows indicate the position on the gel of molecular weight standards. 1, Tissue cytosol; 2, Ammonium sulphate fraction; 3, Ethanol precipitate; 4, DEAE-cellulose; 5, DEAE-Sephadex A-50; 6, Sephadex G-100 (peak III).

Peak III contains a major protein with approximate Mr 35 000 with a few minor contaminants in the Mr 25 000-40 000 range. Analytical gels obtained from other identical purifications always showed a major protein component in the Mr 30 000-35 000 range with some minor contaminants. There are several different molecular weights reported for purified low molecular weight phosphoprotein phosphatases from various tissues although nearly all are in the Mr 30 000-35 000 range (Khandelwal, Vandenheede and Krebs (1976), Khandelwal, Zinman and Ng (1980), Li and Chan (1981), Imazu, Imaoka et al. (1981)). Peak II had a higher protein concentration and much lower specific activity and this was reflected in a more complex pattern on SDS/PAGE. The specific activity of the Mr 35 000 enzyme was 1055 nmol p-nitrophenol produced.min⁻¹.mg protein⁻¹ and 392 pmol ³²P produced.min⁻¹.mg protein⁻¹, representing a purification of 109-fold and 22-fold when measured against p-nitrophenyl phosphate and ³²P-phosphohistone, respectively.

In the purification reported by Li (1979) a final hydrophobic chromatography step using Phenyl-Sepharose 4B was included after the G-100 step. This resulted in an increase in the specific activity of the enzyme when assayed for phosphorylase phosphatase activity but a decrease in specific activity when assayed against p-nitrophenyl phosphate. On two occasions during the purification described here, a further Octyl-Sepharose 4B step was included, but this did not result in an increase in the specific activity when assayed against either substrate and consequently this step was not included routinely.

7.3 STUDIES ON THE MULTIFUNCTIONAL PHOSPHOPROTEIN PHOSPHATASE WITH Mr 35 000 PURIFIED FROM BOVINE ADRENOCORTICAL CYTOSOL

The purified phosphoprotein phosphatase used was the peak III enzyme from the Sephadex G-100 step (Fig. 7.1c). This preparation was concentrated 10-fold by pressure ultrafiltration (Amicon YM10 membrane),

dialysed against 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 10% (v/v) glycerol (buffer D^P) and stored at -20°C in this buffer. The enzyme was active when assayed using p-nitrophenyl phosphate as substrate in the presence of Mg²⁺ and 2-mercaptoethanol (see Table 7.1). However the activity of the enzyme using ³²P-labelled phosphohistone as substrate was not dependent upon divalent cations and was dependent upon the amount of enzyme protein (Fig. 7.3). This difference in divalent cation sensitivity of the two activities was similar to that found by Li (1979).

The source of CEH enzyme used in the following experiments was the cholate solubilised enzyme (see Section 3.3.4), following dialysis against 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 10% (v/v) glycerol. The enzyme was less stable in this buffer upon storage and the subsequent freeze-thawing, and so was stored at -20°C in small batches for no longer than 1 week. This preparation contained endogenous phosphatase activity and so some preliminary experiments were performed to determine the extent of this activity.

7.3.1 Endogenous phosphoprotein phosphatase activity in the partially purified CEH preparation

Samples of the dialysed cholate solubilised enzyme (CEH preparation) were incubated with Mg²⁺ or Mn²⁺, at various concentrations, for 15 min at 37°C. Cholesterol [1-¹⁴C] oleate substrate emulsion was then added and the CEH activity determined as described in the methods section.

Fig. 7.4 shows that with Mg²⁺ there was no deactivation below a concentration of 1 mM, but above this concentration there was an almost linear deactivation with increasing Mg²⁺ concentration, with 30 mM Mg²⁺ giving 50% deactivation. However, Mn²⁺ stimulated the deactivation of CEH at concentrations between one and two orders of magnitude lower than that for Mg²⁺. At concentrations of up to 0.1 mM Mn²⁺, virtually no deactivation was observed but 0.5 mM Mn²⁺ deactivated CEH by 86%.

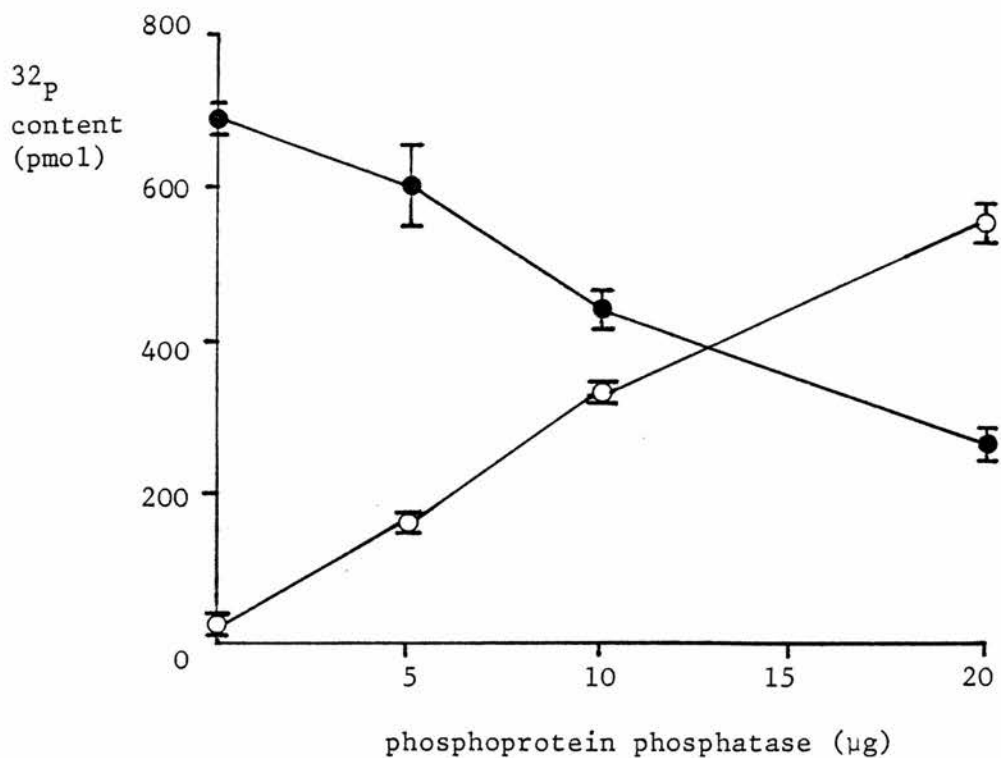


Fig. 7.3. Activity of the Mr 35 000 phosphoprotein phosphatase purified from bovine adrenocortical cytosol when assayed against ^{32}P -labelled phosphohistone. Phosphoprotein phosphatase (0.1 mg ml^{-1} in buffer D^{P}) was incubated with ^{32}P -labelled phosphohistone for 30 min at 37°C . The phosphoprotein phosphatase activity was measured as described in the methods section. Points are means \pm S.E.M. of triplicate assays. O, ^{32}P in TCA supernatant; ●, ^{32}P in TCA precipitated pellet.

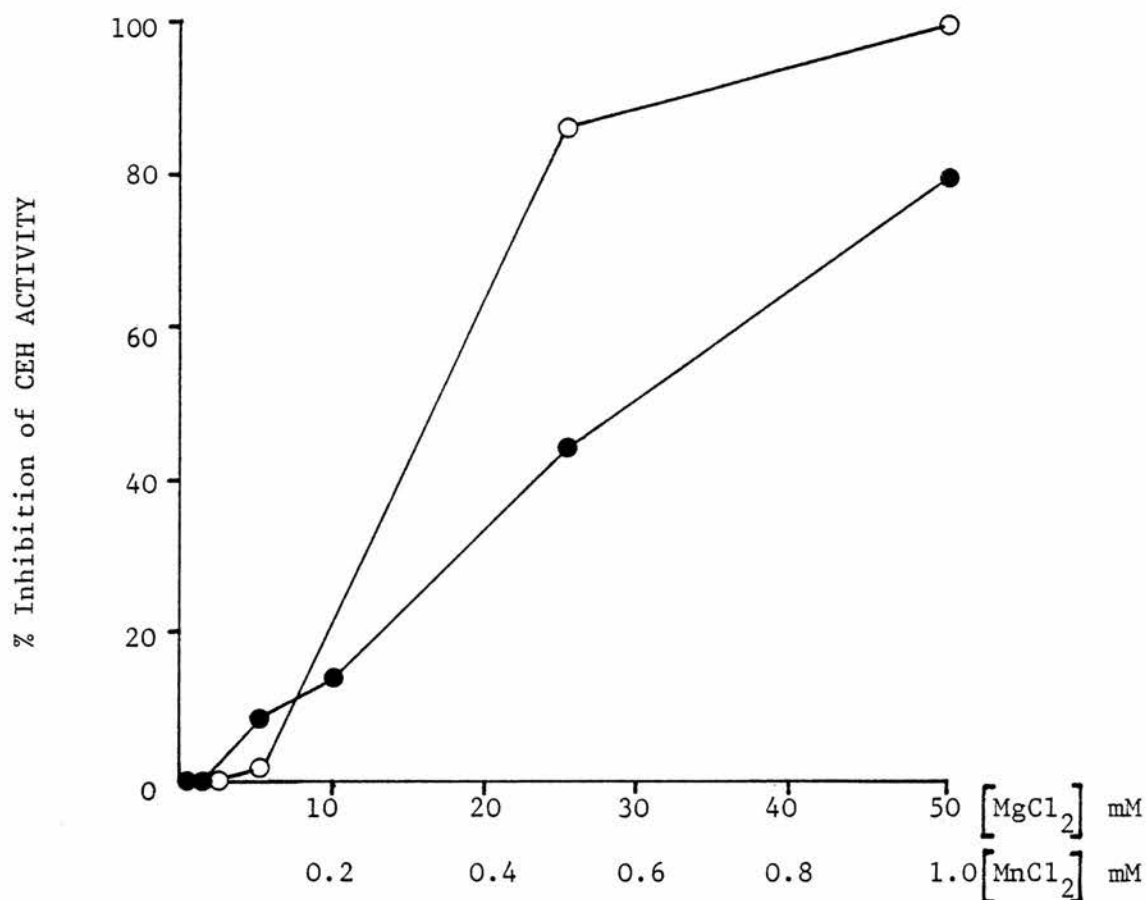


Fig. 7.4. Endogenous $\text{Mg}^{2+}/\text{Mn}^{2+}$ -stimulated phosphoprotein phosphatase activity in the partially-purified CEH preparation. The CEH preparation was the dialysed cholate solubilised enzyme (1 mg ml^{-1}) in 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 10% (v/v) glycerol. Samples ($150 \mu\text{l}$) of this preparation were preincubated for 15 min at 37°C with various concentrations of magnesium or manganese chloride (both in 50 mM Tris-HCl buffer pH 7.4 at 37°C). Cholesterol [$1\text{-}^{14}\text{C}$] oleate substrate emulsion was then added and CEH activity measured as described in the methods section.

○, Mn^{2+} ; ●, Mg^{2+} .

A second preliminary experiment was performed to compare the activity of partially purified multifunctional phosphoprotein phosphatase with the activity of the $\text{Mg}^{2+}/\text{Mn}^{2+}$ -stimulated endogenous phosphoprotein phosphatases, at low Mg^{2+} concentrations. The multifunctional phosphoprotein phosphatase was purified to the stage where the low molecular weight (M_r 35 000) catalytic subunit is released from the holoenzyme, the ethanol precipitate (Step 3). The CEH preparation was incubated with various amounts of Step 3 phosphoprotein phosphatase in buffer A^P , or with buffer A^P alone, for 15 min at 37°C . Cholesterol $[1-^{14}\text{C}]$ oleate substrate was then added and CEH activity measured as before. Buffer A^P contains 5 mM magnesium chloride and thus this experiment showed the effect of low concentrations of Mg^{2+} (0.5–2.0 mM) on CEH activity, in the presence or absence of partially purified phosphoprotein phosphatase.

Fig. 7.5 shows that in this experiment, Mg^{2+} up to a final concentration of 2 mM did not affect the activity of CEH. However the partially purified phosphoprotein phosphatase deactivated the CEH despite the low concentration of Mg^{2+} , and gave 89% deactivation at the highest concentration used. The amount of partially purified phosphoprotein phosphatase added to the incubations increased in parallel with the Mg^{2+} and so this experiment could not give any indication of the ion requirements of the low molecular weight phosphoprotein phosphatase at this stage of the purification.

The two preliminary experiments indicated that the endogenous phosphoprotein phosphatases in the CEH preparation were not stimulated at concentrations of Mg^{2+} and Mn^{2+} below about 2 mM and 0.1 mM, respectively. However, the partially purified multifunctional phosphoprotein phosphatase deactivated CEH at concentrations of Mg^{2+} well below the Mg^{2+} concentration required to activate the endogenous phosphoprotein phosphatases.

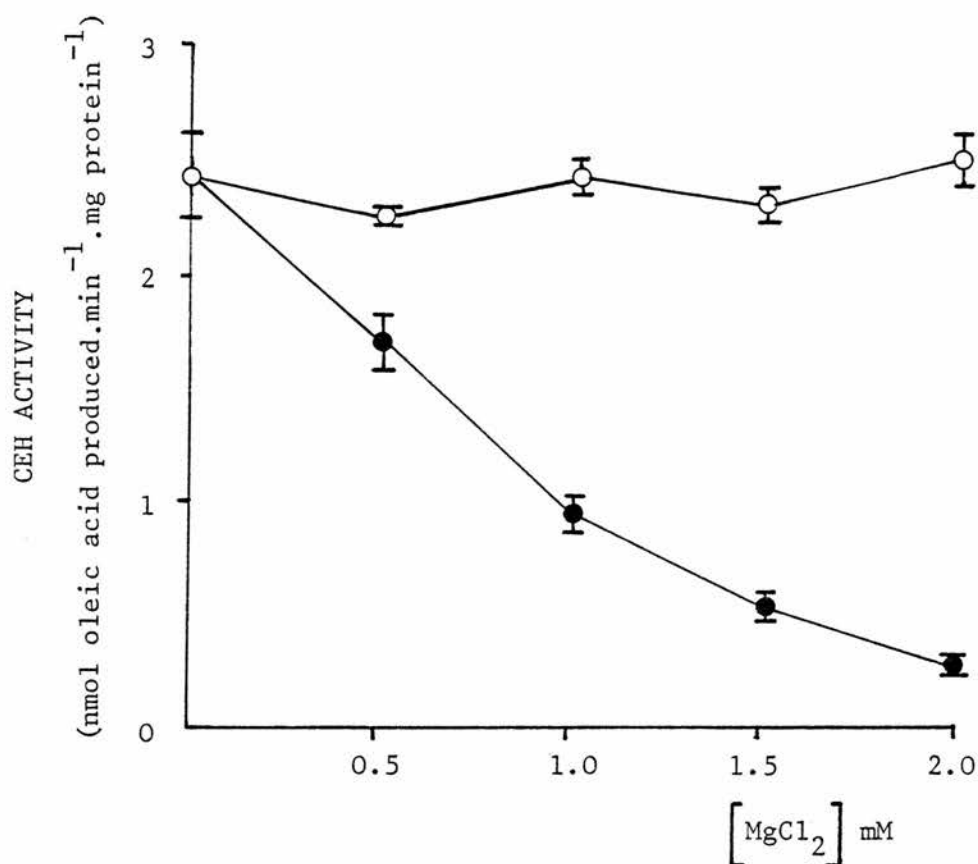


Fig. 7.5. The effect of partially-purified multifunctional phosphoprotein phosphatase on CEH activity at low Mg^{2+} concentrations. The phosphoprotein phosphatase was the step 3 (ethanol precipitate) fraction in buffer A^{P} , from the purification procedure summarised in Table 7.1. The CEH preparation was the cholate solubilised enzyme dialysed against 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 10% (v/v) glycerol. 100 μl samples of CEH preparation (1 mg ml^{-1}) were incubated for 15 min at 37°C with various amounts (25, 50, 75 and 100 μl) of buffer A^{P} , or step 3 phosphoprotein phosphatase in buffer A^{P} . Cholesterol [$1\text{-}^{14}\text{C}$]oleate substrate emulsion was then added and CEH activity measured as described in the methods section. Points are means \pm S.E.M. of triplicate assays.

○, Buffer A^{P} ; ●, step 3 phosphoprotein phosphatase in buffer A^{P} .

7.3.2 The effect of purified multifunctional phosphoprotein phosphatase on CEH activity and ^{32}P -labelled phospho-CEH

All subsequent experiments were carried out using the purified phosphoprotein phosphatase (Mr 35 000) obtained from Step 6 (peak III). The CEH preparation was activated before incubation with the phosphoprotein phosphatase. A sample of CEH preparation was incubated with cAMP, Mg^{2+} and ATP for 10 min at 37°C , desalted on a Sephadex G-25 column and incubated with the phosphoprotein phosphatase, in the presence or absence of 0.1 mM Mn^{2+} , for 15 min at 37°C . Cholesterol $[1\text{-}^{14}\text{C}]$ oleate substrate emulsion was then added and CEH activity measured as described in the methods section. A parallel experiment was performed in which $[\gamma\text{-}^{32}\text{P}]$ ATP replaced ATP in the activation step. After desalting and incubation with phosphoprotein phosphatase as above, the phosphoprotein phosphatase activity was assayed as described in the methods section.

Table 7.2 shows that the purified phosphoprotein phosphatase contained no CEH activity and that the activation of CEH by endogenous cAMP-dependent protein kinase resulted in a 63% increase in the CEH activity. Incubation with 0.1 mM Mn^{2+} resulted in a reduction in the CEH activity and an increase in ^{32}P released from the protein pellet, however these differences were not significant ($p > 0.1$). This was as predicted from the preliminary experiments where this concentration of Mn^{2+} did not stimulate endogenous phosphoprotein phosphatases (Fig. 7.4). Addition of the phosphoprotein phosphatase, in the absence of Mn^{2+} , had contrasting effects in the parallel experiments. The phosphoprotein phosphatase caused a 65% increase in the ^{32}P released into the TCA supernatant and a corresponding 46% decrease in the ^{32}P content of the protein fraction. However the phosphoprotein phosphatase had no significant effect upon the activity of CEH. Addition of 0.1 mM Mn^{2+} to the incubations with phosphoprotein phosphatase had no significant effect on either of these

Sample	Phosphoprotein Phosphatase Activity		CEH Activity (pmol oleic acid produced.min ⁻¹ .mg protein ⁻¹)
	³² P in Supernatant (pmol)	³² P in Pellet (pmol)	
Basal CEH activity	-	-	1569 ± 46
CEH activity in phosphoprotein phosphatase	-	-	0
<u>Activated CEH preparation</u>			
Alone	41.0 ± 0.6	43.0 ± 1.6	2552 ± 200
+ 0.1 mM Mn ²⁺	42.6 ± 0.2	40.0 ± 2.0	2290 ± 100
+ phosphoprotein phosphatase	67.6 ± 0.6	23.4 ± 2.2	2447 ± 62
+ phosphoprotein phosphatase and 0.1 mM Mn ²⁺	71.4 ± 0.8	23.6 ± 1.8	2589 ± 208

Table 7.2. Effect of multifunctional phosphoprotein phosphatase on CEH activity and ³²P-labelled phosphoCEH. The CEH preparation (1 mgml⁻¹) was incubated for 10 min at 37°C with 5 mM magnesium chloride, 20 μM cAMP and 5 mM ATP or 0.2 mM [γ-³²P]ATP. The samples were desalted on a Sephadex G-25 column equilibrated in buffer D^P. The desalted samples were incubated for 15 min at 37°C with or without purified phosphoprotein phosphatase (10 μg in buffer D^P) in the presence or absence of 0.1 mM Mn²⁺. The phosphoprotein phosphatase and CEH activities were measured as described in the methods section. Results are expressed as means ± S.E.M. of triplicate assays.

results. The activity of the purified phosphoprotein phosphatase towards the ^{32}P -labelled proteins in the CEH preparation was dependent upon the amount of phosphoprotein phosphatase enzyme (Fig. 7.6). The specific activity of the phosphoprotein phosphatase when measured against this substrate was $19 \text{ pmol } ^{32}\text{P released} \cdot \text{min}^{-1} \cdot \text{mg enzyme}^{-1}$.

These results indicated that the multifunctional phosphoprotein phosphatase was of low specificity and divalent cation-independent when assayed against various ^{32}P -labelled proteins in the CEH preparation. However this enzyme appeared to be unable to deactivate the CEH enzyme.

7.3.3 Further studies on the effect of the multifunctional phosphoprotein phosphatase on CEH activity

The effect of a higher concentration of Mn^{2+} and the effect of Mg^{2+} on the activity of the phosphoprotein phosphatase, when measured towards CEH activity, was investigated. Samples of CEH preparation were incubated with the phosphoprotein phosphatase for 15 min at 37°C in the presence or absence of various concentrations of Mn^{2+} or Mg^{2+} . Controls with Mg^{2+} and Mn^{2+} alone were also included. After incubation, cholesterol [$1\text{-}^{14}\text{C}$] oleate substrate emulsion was added and the CEH activity determined.

Table 7.3 shows that the phosphoprotein phosphatase alone did not deactivate CEH. Mg^{2+} at 1 mM and 10 mM deactivated the enzyme by 15% and 76% respectively. Addition of the phosphoprotein phosphatase did not significantly alter these results ($p > 0.05$). Mn^{2+} at 1 mM was sufficient to reduce the CEH activity to zero and addition of phosphoprotein phosphatase had no effect. However at 0.1 mM, Mn^{2+} alone resulted in 96% deactivation of CEH. This was far greater than experienced with previous CEH preparations and suggests this preparation, although prepared identically, contained a more active endogenous phosphoprotein phosphatase. The deactivation with Mg^{2+} alone was also greater than observed previously (Fig. 7.4). Furthermore, the deactivation

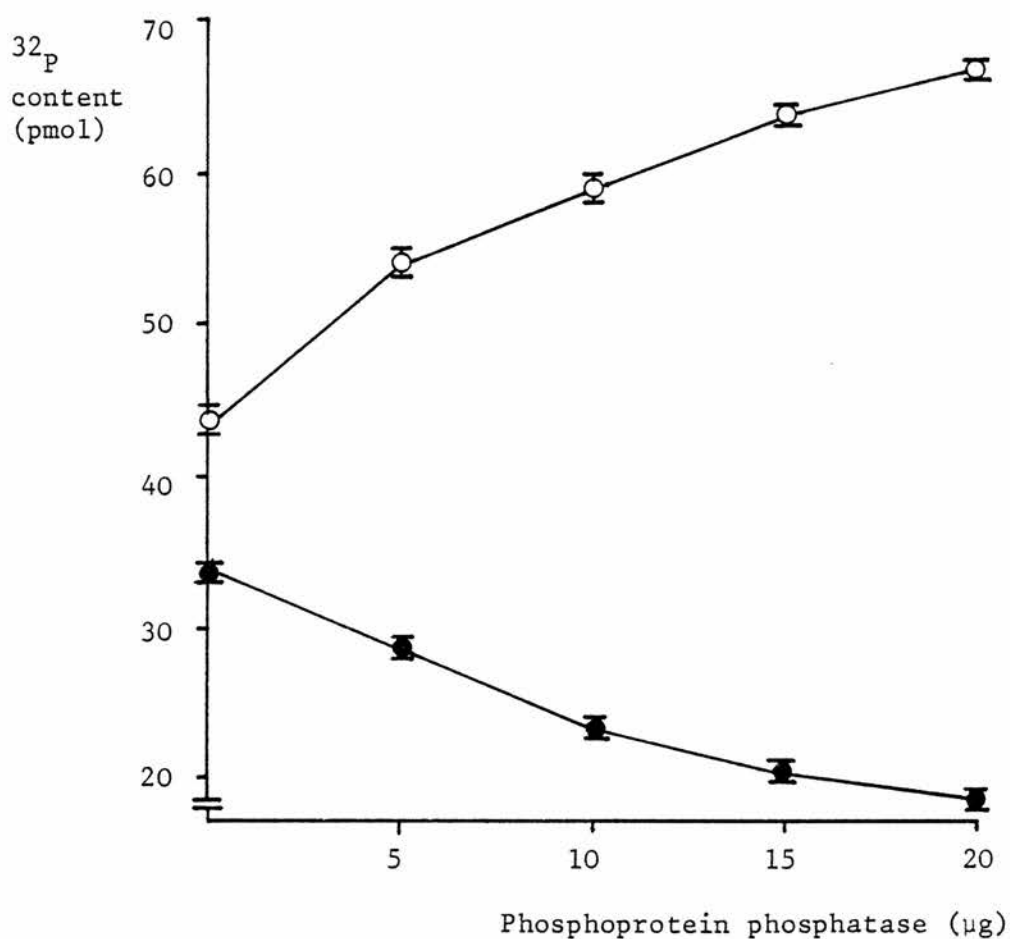


Fig. 7.6. Activity of the multifunctional phosphoprotein phosphatase against ^{32}P -labelled proteins in the CEH preparation. The experiment was performed as described in the legend to Fig. 7.3 except that the phosphoprotein phosphatase substrate was the ^{32}P -labelled CEH preparation prepared as described in the legend to Table 7.2.

Points are means \pm S.E.M. of triplicate assays.

O, ^{32}P in TCA supernatant; ●, ^{32}P in TCA precipitate.

Additions to CEH Preparation			CEH Activity
Phosphoprotein Phosphatase (μg)	Mn ²⁺ (mM)	Mg ²⁺ (mM)	(pmol oleic acid produced.min ⁻¹ mg protein ⁻¹)
-	-	-	1263 \pm 14
7	-	-	1393 \pm 40
-	0.1	-	49 \pm 32
7	0.1	-	1162 \pm 87
-	1.0	-	0
7	1.0	-	0
-	-	1.0	1076 \pm 30
7	-	1.0	1278 \pm 90
-	-	10.0	306 \pm 45
7	-	10.0	265 \pm 31

Table 7.3. The effect of Mn²⁺ and Mg²⁺ on the activity of the multifunctional phosphoprotein phosphatase when assayed against CEH activity. Dialysed cholate solubilised CEH preparation (0.43 mgml⁻¹ in buffer D^P) was incubated for 15 min at 37°C with the additions indicated above. The phosphoprotein phosphatase (35 μgml^{-1}) was in buffer D^P and Mn²⁺ and Mg²⁺ were added as the chloride salts in distilled water. After incubation cholesterol [1-¹⁴C] oleate substrate emulsion was added and CEH activity assayed as described in the methods section. Results are expressed as means \pm S.E.M. of triplicate assays.

by 0.1 mM Mn^{2+} was inhibited by the addition of the purified phosphoprotein phosphatase. This suggested that the phosphoprotein phosphatase was chelating the Mn^{2+} at this low concentration and thus preventing the Mn^{2+} from stimulating the endogenous phosphoprotein phosphatases. A similar effect was observed with 1 mM Mg^{2+} .

The results have shown that the multifunctional phosphoprotein phosphatase alone was not able to deactivate CEH. When concentrations of Mn^{2+} or Mg^{2+} that stimulate endogenous phosphoprotein phosphatases are included in the incubations, no increased deactivation was observed upon the addition of the multifunctional phosphoprotein phosphatase.

The alkaline phosphatase activity of the Mr 35 000 enzyme required a thiol such as 2-mercaptoethanol or DTT for full activity (Li (1979)). Consequently, an experiment was performed in which the CEH preparation was incubated with the phosphoprotein phosphatase and 1 mM or 5 mM Mg^{2+} in the presence or absence of 1 mM DTT. Table 7.4 shows that 1 mM Mg^{2+} caused a 10% reduction in CEH activity and this was not significantly altered by the addition of DTT or DTT plus phosphoprotein phosphatase ($p > 0.1$). With 5 mM Mg^{2+} , the CEH activity was reduced by 54% and the addition of DTT or DTT plus phosphoprotein phosphatase tended to inhibit the activity of the endogenous phosphoprotein phosphatases. The reason for the latter result was unknown, but the results show that the activity of the phosphoprotein phosphatase towards CEH activity was not stimulated by a thiol.

It had been shown that the metal ionⁱⁿ-dependent form of the Mr 35 000 enzyme could be converted by pyrophosphoryl compounds (e.g. ATP or PP_i) to a metal ion-dependent form which, subsequently, could be reactivated by incubation with Co^{2+} or Mn^{2+} but not with Mg^{2+} (Li (1979)). It was possible that the phosphoprotein phosphatase was converted to this form of the enzyme during the purification procedure, particularly during

Additions to CEH Preparation			CEH Activity
Mg ²⁺ (mM)	DTT (mM)	Phosphoprotein Phosphatase (μg)	(pmol oleic acid produced.min ⁻¹ . mg protein ⁻¹)
-	-	-	1458 ± 36
1.0	-	-	1322 ± 88
1.0	1.0	-	1251 ± 34
1.0	1.0	7	1318 ± 30
5.0	-	-	671 ± 33
5.0	1.0	-	883 ± 24
5.0	1.0	7	1063 ± 7

Table 7.4. Effect of DTT on the activity of the multifunctional phosphoprotein phosphatase when assayed against CEH activity.

The CEH preparation, phosphoprotein phosphatase and Mg²⁺ were all as described in the legend to Table 7.3. DTT was in 20 mM Tris-HCl buffer pH 7.4 at 37°C. The experiment was performed as described in the legend to Table 7.3. Results are expressed as means ± S.E.M. of triplicate assays.

the preparation of cytosol when pyrophosphoryl compounds would be present. After this stage, however, there were several dialysis steps that would remove pyrophosphoryl compounds from the preparation. Furthermore the activity of the enzyme, when assayed against ^{32}P -labelled phosphohistone would be low and this was not observed (see Table 7.1 and Fig. 7.3).

Despite this evidence, as there was a possibility of the enzyme existing in a less active form, samples of the phosphoprotein phosphatase were incubated for 20 min at 4°C with 0.1 mM Mn^{2+} and 0.01 mM Co^{2+} .

The studies by Li (1979) had shown that half maximal activation of the pyrophosphate-inactivated enzyme could be achieved with $80\text{ }\mu\text{M Mn}^{2+}$ or $5\text{ }\mu\text{M Co}^{2+}$. The preincubated phosphoprotein phosphatase/ion mixtures were then assayed for activity towards CEH activity as in the previous experiments. In the controls, the CEH preparation was incubated with $40\text{ }\mu\text{M Mn}^{2+}$ and $4\text{ }\mu\text{M Co}^{2+}$ alone. This was the concentration of the ions in the CEH/phosphoprotein phosphatase incubations.

Table 7.5 shows that incubation of the CEH preparation with $40\text{ }\mu\text{M Mn}^{2+}$ had no significant effect on CEH activity, but when the CEH preparation was incubated with the phosphoprotein phosphatase/ Mn^{2+} mixture there was a 31% increase in the CEH activity, which was significant ($p < 0.02$). Similarly, incubation of the CEH preparation with Co^{2+} resulted in a slight but not significant increase in CEH activity ($p > 0.1$) but there was a 47% increase in CEH activity when the CEH preparation was incubated with the phosphoprotein phosphatase/ Co^{2+} mixture ($p < 0.001$).

The mechanisms involved in these increases in CEH activity were unknown. The phosphoprotein phosphatase/ Mn^{2+} result was different to any obtained in previous experiments. Table 7.3 showed that, at low concentrations, all the Mn^{2+} was chelated by the phosphoprotein phosphatase and so a significant increase in activity was difficult

Additions to CEH Preparation				CEH Activity
Mn ²⁺ (μ M)	Co ²⁺ (μ M)	Phosphoprotein ₂ ⁺ Phosphatase/Mn ²⁺ (μ g, μ M)	Phosphoprotein ₂ ⁺ Phosphatase/Co ²⁺ (μ g, μ M)	(pmol oleic acid produced.min ⁻¹ .mg protein ⁻¹)
-	-	-	-	1069 \pm 50
40	-	-	-	1119 \pm 14
-	-	(7,40)	-	1406 \pm 62
-	4	-	-	1222 \pm 95
-	-	-	(7,4)	1572 \pm 12

Table 7.5. The effect of preincubation with Co²⁺ and Mn²⁺ on the activity of the multifunctional phosphoprotein phosphatase when assayed against CEH activity. Samples of the purified phosphoprotein phosphatase were preincubated with 0.1 mM Mn²⁺ or 0.01 mM Co²⁺ for 20 min at 4°C. Mn²⁺ and Co²⁺ were the chloride salts in distilled water. The preincubated phosphoprotein phosphatase/ion mixtures, and controls of ions alone, were incubated with CEH preparation and the experiment completed as described in the legend to Table 7.3. Results are expressed as means \pm S.E.M. of triplicate assays.

to explain. No work on the effect of Co^{2+} on the activity of CEH had been reported, but it appeared that Co^{2+} alone may stimulate CEH activity. How preincubation of Co^{2+} with phosphoprotein phosphatase could stimulate this activation was not known.

Another ion that may be involved in metabolic regulation such as this is Ca^{2+} . In the study of the 35 000 Mr phosphoprotein phosphatase from bovine adrenal cortex by Li (1979), Ca^{2+} was found to have no effect on the activity of the enzyme towards phosphohistone and phosphocasein. In equimolar concentrations it could protect the enzyme from inactivation by pyrophosphoryl compounds, but could not reactivate the inactivated form, nor could it serve as an activator for the alkaline phosphatase activity. However it was possible that Ca^{2+} could have an effect on the activity of the enzyme towards CEH, consequently various concentrations of Ca^{2+} were incubated with the CEH preparation in the presence and absence of phosphoprotein phosphatase. Intracellular Ca^{2+} concentration in the cytosol of most mammalian cells is about 10^{-8} - 10^{-7} M, rising to about 10^{-6} M in 'stimulated cells' (Cheung (1980)). Therefore a series of Ca^{2+} concentrations in this range and up to 1 mM was chosen for this experiment.

Fig. 7.7 shows that Ca^{2+} up to 10^{-5} M had no effect on CEH activity, but at higher concentrations Ca^{2+} inhibited CEH. This may be a direct effect on the CEH enzyme or it may be due to a stimulation of endogenous phosphoprotein phosphatases. Inclusion of phosphoprotein phosphatase had no effect up to 10^{-5} M Ca^{2+} , and at higher concentrations it partly inhibited the Ca^{2+} -induced reduction, but not significantly.

The results in this section strongly suggest that the multifunctional phosphoprotein phosphatase was not able to deactivate CEH.

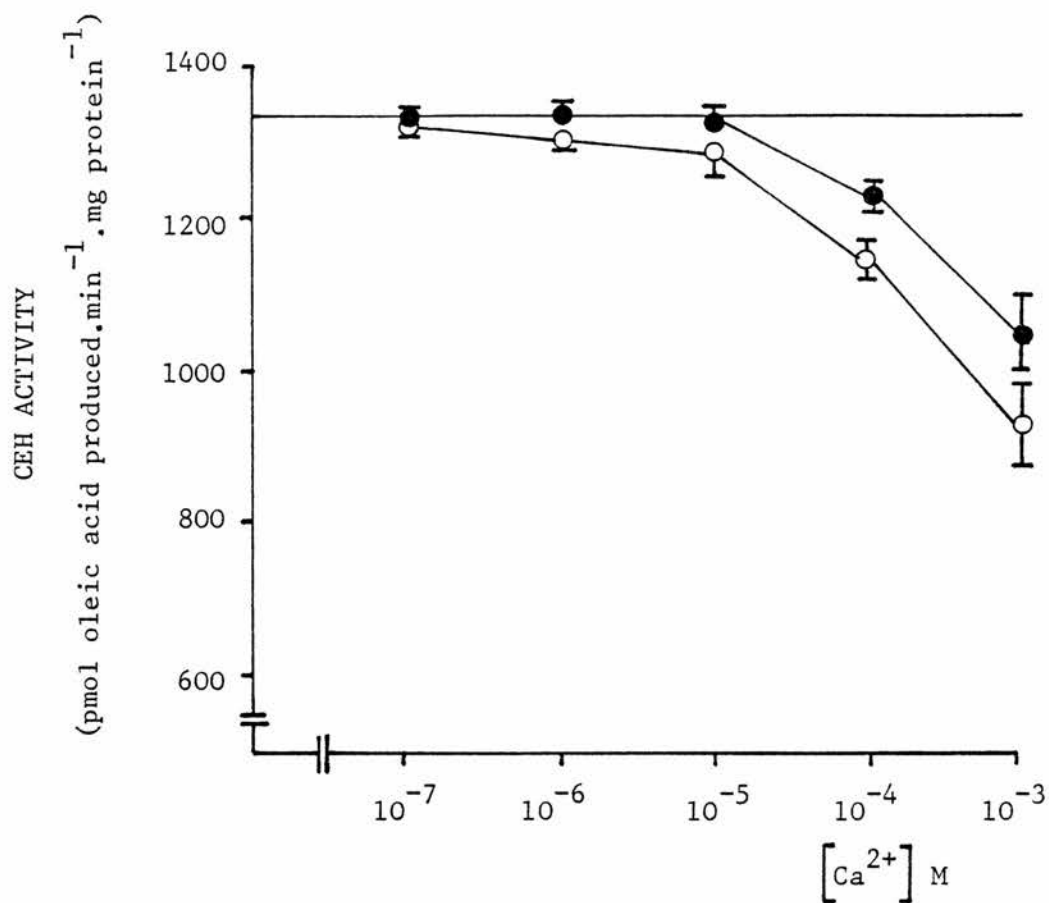


Fig. 7.7. Effect of Ca^{2+} on the activity of CEH and the multifunctional phosphoprotein phosphatase. The CEH preparation and the purified phosphoprotein phosphatase were as described in the legend to Table 7.3. Ca^{2+} was the chloride salt in distilled water. The CEH preparation was incubated for 15 min at 37°C with either Ca^{2+} or Ca^{2+} plus phosphoprotein phosphatase (10 μg) at the Ca^{2+} concentrations indicated. Cholesterol $[1\text{-}^{14}\text{C}]$ oleate substrate emulsion was then added and CEH activity measured as described in the methods section. Points are means \pm S.E.M. of triplicate assays.

—, Control CEH activity (no additions); O, Ca^{2+} ; ●, Ca^{2+} plus phosphoprotein phosphatase.

7.4 PURIFICATION OF A LOW MOLECULAR WEIGHT PHOSPHOPROTEIN PHOSPHATASE USING DEACTIVATION OF CEH AS THE ASSAY

It was possible that during the purification of the enzyme, using p-nitrophenyl phosphate and ^{32}P -labelled phosphohistone as substrates to monitor the phosphatase activity, a fraction active against CEH was discarded. This appeared possible as the results shown in Fig. 7.5 indicate that there was a phosphoprotein phosphatase activity in the ethanol precipitate that was active against CEH in the presence of low concentrations of Mg^{2+} . Therefore the purification was repeated using CEH deactivation as the assay and pooling the fractions that deactivate CEH.

The CEH preparation used as the substrate in this assay procedure was the cholate solubilised enzyme after dialysis into buffer D^{P} . Therefore, this preparation contained Mg^{2+} -stimulated endogenous phosphoprotein phosphatases (Fig. 7.4). The main buffer (buffer A^{P}) used in the phosphoprotein phosphatase purification contained 5 mM Mg^{2+} and 1 mM EDTA, thus in an assay incubation with equal volumes of CEH preparation and column fraction the Mg^{2+} concentration may be 2 mM. This may stimulate the endogenous phosphoprotein phosphatases in the CEH preparation sufficiently to mask any additional deactivation due to the phosphoprotein phosphatase activity in the column fractions. Therefore the Mg^{2+} concentration in buffer A^{P} was reduced to 2 mM and EDTA was omitted. The final Mg^{2+} concentration in the assay incubations was now 1 mM, which should reduce activation of endogenous phosphoprotein phosphatases in the CEH preparation to a minimum, and still activate the phosphoprotein phosphatase present in the ethanol precipitate if this was required (Fig. 7.5).

The purification was performed as described in Section 7.2 with the buffer modifications mentioned, except that the column fractions were

assayed for p-nitrophenyl phosphatase activity and CEH deactivating activity (see Fig. 7.8).

Fig. 7.8a shows that p-nitrophenyl phosphatase activity eluted from DEAE-cellulose in 3 peaks, at 0.25 M and 0.35 M potassium chloride as observed previously (Fig. 7.1a), and a further peak that was not adsorbed but was eluted just after the main protein peak. This result had been observed previously, but in this case it was unusually large. The main peak of CEH deactivating activity was not adsorbed and eluted between the main protein peak and the unadsorbed p-nitrophenyl-phosphatase peak. This peak was pooled as indicated in Fig. 7.8a. A smaller CEH deactivating peak was eluted at 0.28 M potassium chloride, but this was not pooled in this purification. This was because the second peak was associated with fractions that had been pooled during the previous purifications (see Fig. 7.1a). In the later fractions from this column the CEH activity was also affected by the high salt concentration.

The pooled material from the DEAE-cellulose step was then applied (without dialysis) to the DEAE-Sephadex A-50 column equilibrated with the modified buffer A^P . The column was developed with a linear gradient of potassium chloride (0.05-0.45 M) in modified buffer A^P . Fig. 7.8b shows that the p-nitrophenyl phosphatase activity eluted in a broad peak between the peaks of unadsorbed and adsorbed protein, and was partly eluted as the gradient was applied. This suggested that the activity was not fully adsorbed but merely retarded by the gel. No CEH deactivating activity was detected in the fractions eluted from this column. It was possible that the phosphoprotein phosphatase was too dilute when eluted from the column to be detected in this particular assay. The fractions indicated in Fig. 7.8b were pooled using the p-nitrophenyl phosphatase activity as a guide, because the CEH deactivating

Legend to Fig. 7.8. Purification of a low molecular weight phosphoprotein phosphatase using deactivation of CEH as an assay.

a) DEAE-cellulose - The column (2.5 x 35 cm) was equilibrated with modified buffer A^P (see text) and eluted with a linear gradient of potassium chloride (0.05-0.45 M) in a total volume of 500 ml. The flow rate was 90 ml.h⁻¹ and fractions of 8.2 ml were collected.

b) DEAE-Sephadex A-50 - The column (2.1 x 25 cm) was equilibrated in modified buffer A^P and eluted with a linear gradient of potassium chloride (0.05-0.45 M) in a total volume of 600 ml. The column operated under a pressure of 25 cm H₂O and fractions of 8.2 ml were collected.

c) Sephadex G-100 - The column (2 x 95 cm) was equilibrated and eluted with buffer C^P under a pressure of 70 cm H₂O and fractions of 5.8 ml were collected.

p-Nitrophenol phosphatase activity was measured as described in the methods section. CEH deactivating activity was measured by incubating 100 µl column fraction for 10 min at 37°C with 100 µl CEH preparation (cholate solubilised enzyme dialysed into buffer D^P, 1.54 mgml⁻¹). 200 µl cholesterol [1-¹⁴C]oleate substrate emulsion was then added and CEH activity determined as described in the methods section.

△, CEH deactivating activity; ●, p-Nitrophenyl phosphatase;

▲, A₂₈₀; ---, Potassium chloride concentration; └─┘, Pooled fractions.

activity was associated with this in the DEAE-cellulose step. This material was concentrated by pressure ultrafiltration (Amicon YM10 membrane) to about 15 ml and dialysed against 20 mM Tris-HCl buffer pH 7.4 at 4°C containing 10 mM 2-mercaptoethanol and 50% glycerol, which resulted in a further concentration to about 6 ml.

The concentrated material was applied to the Sephadex G-100 column equilibrated and eluted with buffer C^P. Fig. 7.8c shows that no CEH deactivating activity was detected in the fractions from this column. A large proportion of the protein applied was eluted in the void volume but there were 2 peaks of protein that were retarded and the p-nitrophenyl phosphatase activity was associated with the second of these. The fractions indicated were pooled and concentrated by pressure ultrafiltration (Amicon YM10 membrane) but no CEH deactivating activity was detected in the concentrated sample. SDS/PAGE of the sample showed a major protein with Mr 20 000, a further protein with Mr 15 000 and minor components with Mr 35 000 and 18 000 (Fig. 7.9).

There were no reports in the literature of a phosphatase with a molecular weight as low as 20 000 or 18 000, although Severson and Sloan (1980) reported a phosphoprotein phosphatase with Mr 28 000, obtained from rat adipose tissue, that was capable of deactivating HSL in the presence of divalent cations. It was possible that the alkaline phosphatase activity (684 pmol p-nitrophenol produced.min⁻¹.mg protein⁻¹) was due to the contaminating 35 000 Mr protein. As no ³²P-labelled phosphohistone was available at that time, it was not known if this preparation exhibited phosphoprotein phosphatase activity. However as the aim of this purification, to purify a CEH deactivating activity, was not achieved, the alkaline phosphatase activity was not studied further.

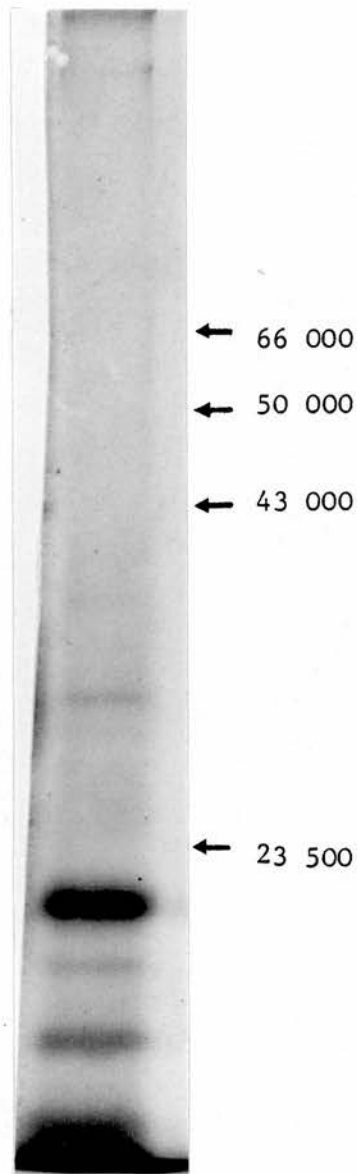


Fig. 7.9. SDS/PAGE of the low molecular weight phosphoprotein phosphatase, purified as described in Section 7.4. A sample (25 μ g protein) of the concentrated, pooled fractions from the Sephadex G-100 chromatography step was subjected to SDS/PAGE on a 10% gel and stained for protein as described in the methods section.

The arrows indicate the position on the gel of molecular weight standards.

The reason for the loss of activity between the DEAE-cellulose and DEAE-Sephadex A-50 steps was not known although overnight storage at 4°C may be responsible (Khandelwal, Vandenheede and Krebs (1976)). It was possible that there was proteolysis by a contaminating protease. Proteolysis was known to be a method for the production of low molecular weight phosphoprotein phosphatases from the high molecular weight forms (Vandenheede, Yang and Merlevede (1981)). However the 35 000 Mr protein was known to be resistant to proteolysis (Mellgren, Aylward et al.(1979)), suggesting that the 20 000 and 15 000 Mr proteins were not likely to arise by proteolysis of the 35 000 Mr protein.

The results from all the experiments suggest that the 35 000 Mr multifunctional phosphoprotein phosphatase was not responsible for the deactivation of CEH. The 35 000 Mr subunit may require another subunit, present in the physiological (high molecular weight) form of the phosphoprotein phosphatase, to gain access to and dephosphorylate an enzyme of a lipoprotein nature, such as CEH. The amounts of phospholipid or cholate that remained associated with CEH in the dialysed cholate solubilised enzyme preparation were not known. However the results in Section 7.4 showed that the CEH deactivating activity present in the ethanol precipitate was lost during the subsequent purification steps.

7.5 SUMMARY

1. The low molecular weight (Mr 35 000) multifunctional phosphoprotein phosphatase was purified from bovine adrenocortical cytosol.
2. This phosphoprotein phosphatase was active when assayed against ^{32}P -labelled phosphohistone, ^{32}P -labelled phosphoCEH preparation and p-nitrophenyl phosphate. Divalent cations were not required for the phosphoprotein phosphatase activity but Mg^{2+} and a thiol were required for the alkaline phosphatase activity.

3. The 35 000 Mr phosphoprotein phosphatase did not deactivate CEH.
4. Addition of Mg^{2+} , Mn^{2+} , Co^{2+} , Ca^{2+} or DTT did not stimulate the phosphoprotein phosphatase to deactivate CEH.
5. During preparation of the 35 000 Mr phosphoprotein phosphatase the ability to deactivate CEH was lost at a stage after ethanol precipitation.
6. Possible explanations for these observations were discussed.

SECTION 8

IN VIVO REGULATION OF RAT ADRENAL CYTOSOLIC CHOLESTEROL

ESTER HYDROLASE ACTIVITY

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8.1 INTRODUCTION

Cholesterol is the sole precursor for steroidogenesis and is stored in the adrenal cortex in lipid droplets mainly in the form of long chain fatty acid esters (Moses, Davis et al.(1969)). It had been shown that administration of ACTH to hypophysectomised rats caused a depletion in the cholesterol ester concentration of the adrenals with a concomitant increase in the corticosteroid output (Davis and Garren (1966)). Later it was shown that the activity of CEH could be enhanced by ACTH administration to intact (Behrman and Greep (1972)) or hypophysectomised (Shima, Mitsunaga and Nakao (1972)) rats, or by increasing the concentration of corticotropin in the blood by ether anaesthesia stress (Trzeciak and Boyd (1973)). It was later established that ACTH caused an increase in intracellular cAMP which led to the phosphorylation and activation of cytosolic CEH with subsequent hydrolysis of cholesterol esters in the lipid droplets and an increase in corticosteroid output (see Boyd and Gorban (1980)).

It has been shown that ACTH secretion, in all animal species investigated, exhibits a diurnal variation and that the glucocorticoid output from the adrenals reflects the variation in ACTH secretion (Boissin, Nauguier-Soulé and Assenmacher (1976)). In a nocturnal animal such as the rat, ACTH secretion rises during the light, resting phase to a maximum at the onset of the dark, waking phase, before falling back to a minimum at the onset of the light phase (Retiene, Zimmerman et al. (1968)). The serum corticosterone concentration correlates with this, displaying a similar variation through the light-dark cycle (Retiene, Zimmerman et al.(1968), Ixart, Szafarczyk et al.(1977)). It had also been shown that adrenal total cholesterol esters undergo a diurnal variation, with the level falling from the middle of the light phase to a minimum in the middle of the dark phase before rising to a maximum in the middle of the light phase (Young and Walker (1978)).

Despite the known involvement of CEH between ACTH stimulation and glucocorticoid output (see Figs. 1.1 and 1.3), little work had been carried out to investigate the variation of CEH activity during a 12 h light-12 h dark period. Previous work had shown that the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the enzyme that regulates cholesterol biosynthesis, varies diurnally in the adrenal gland (Balasubramaniam, Goldstein and Brown (1977)). However, Pederson and Brownie (1979) measured CEH activity in rat adrenals at two time points, 10 h apart, but found no difference in the enzyme activity.

The experiments described in this section were performed to investigate the activity of adrenal cytosolic CEH throughout a 24 h period in rats adapted to a 12 h light-12 h dark cycle, to discover if there was a diurnal variation in the enzyme activity. The serum corticosterone concentration was also measured in these experiments. The effects of acute administration of ACTH and corticoid treatment were investigated to assess the role of ACTH in the production and maintenance of any enzyme variation.

8.2 DIURNAL VARIATION IN CYTOSOLIC CEH ACTIVITY IN THE RAT ADRENAL

Adrenal cytosolic CEH activity over a 24 h period was measured in rats. The animals were kept in a 12 h dark-12 h light cycle and groups were killed at intervals of 6 h as described in the methods section. The adrenals were collected, tissue cytosol prepared and CEH activity measured as described in the methods section. Trunk blood was also collected for serum corticosterone measurements as described in the methods section.

The results in Fig. 8.1 showed that rat adrenal CEH exhibited a diurnal variation in activity with the peak occurring during the dark phase. The serum corticosterone concentration showed a similar variation, with the peak occurring during the dark phase. The differences between

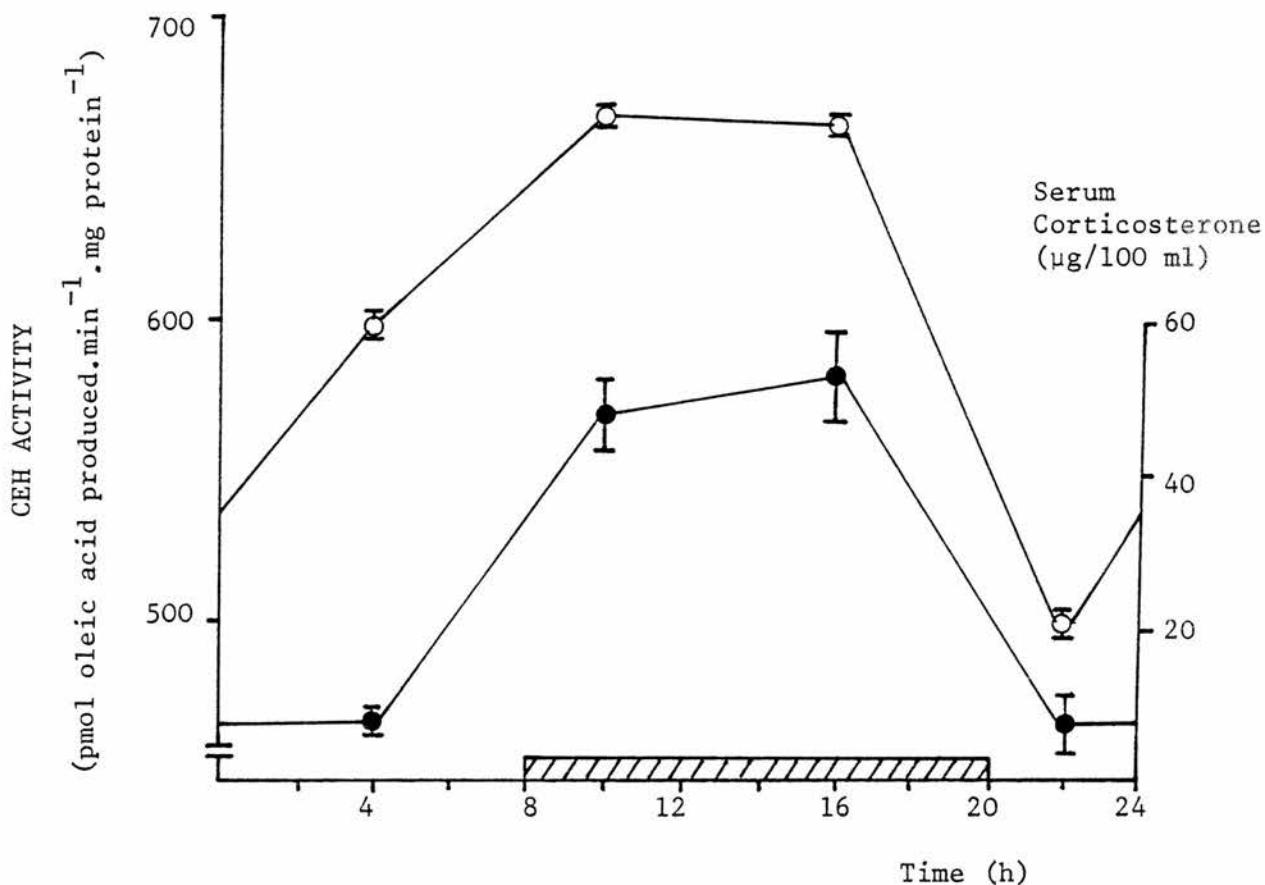


Fig. 8.1. Diurnal variation in rat adrenal cytosolic CEH activity and serum corticosterone concentration. Rats adapted to a 12 h light- 12 h dark cycle (as indicated) were killed at 6 h intervals. Blood was collected for serum corticosterone assays (●) and tissue cytosol was prepared from the adrenals for CEH activity measurements (○).

Details of the experiment are given in the methods section. For CEH activity the points are means \pm S.E.M. for 18 assays. For serum corticosterone the points are mean values obtained from 6 rats \pm S.E.M.

▨, Dark phase.

the values obtained during the dark phase and the values obtained during the light phase were highly significant ($p < 0.001$) for both CEH activity and serum corticosterone. However there was no significant difference between the two measurements made during the dark phase at 10.00 h and 16.00 h for the CEH activity or serum corticosterone.

In a series of four experiments this rhythmic variation was observed on each occasion (Table 8.1). For the CEH activity figures all the differences between the values obtained during the dark phase and the values obtained during the light phase were highly significant ($p < 0.001$, except for experiment 3 10.00 h-04.00 h, $p < 0.05$). In all but one instance the values obtained during the light phase were lower than those during the dark phase. The exception to this was the figure obtained at 22.00 h in experiment 2. This was exceptionally high, and considerably higher than any other activity obtained during these experiments. The reason for this was not known but it was possible that this group of animals were stressed in some way, prior to killing. However, the serum corticosterone concentrations for this experiment do not support this theory, as the value obtained at 22.00 h was very much lower than the values obtained during the dark phase and not significantly different to the value obtained at 04.00 h during the light phase. As all the values obtained in this experiment were higher than in the other three experiments, it was possible that substrate variation was responsible for the differences observed in this experiment.

Despite this one opposing figure, the results show a clear diurnal variation for CEH activity, with the enzyme significantly more active in the dark phase. On two occasions (experiments 1 and 4) there was no significant difference between the two values for CEH activity obtained during the dark phase. In the other two experiments the enzyme activity was higher in the early part of the dark phase in one experiment and

A. CEH Activity (pmol oleic acid produced.min⁻¹.mg protein⁻¹)

Expt.	No. of rats	Time (h)			
		04.00	10.00	16.00	22.00
1	24	604 ± 4	668 ± 3	664 ± 3	499 ± 4
2	24	896 ± 2	986 ± 10	1055 ± 9	1229 ± 5
3	24	525 ± 6	542 ± 4	574 ± 5	518 ± 5
4	12	569 ± 3	652 ± 5	641 ± 12	542 ± 6



B. Serum Corticosterone (µg/100 ml)

Expt.	No. of rats	Time (h)			
		04.00	10.00	16.00	22.00
1	24	8.0 ± 1.5	47.3 ± 4.3	53.1 ± 6.4	7.1 ± 4.6
2	24	16.4 ± 6.2	68.1 ± 10.1	53.3 ± 3.0	16.7 ± 5.0
3	24	56.1 ± 2.2	87.5 ± 2.5	82.1 ± 9.5	29.7 ± 5.8
4	12	7.7 ± 2.4	20.3 ± 5.7	15.6 ± 3.6	16.4 ± 4.2

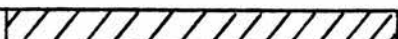



Table 8.1. Diurnal variation in rat adrenal cytosolic CEH activity and serum corticosterone concentration. The experiment was performed and the results expressed as described in the legend to Fig. 8.1.

 indicates results obtained during dark phase.

higher in the later part of the dark phase in the other. Apart from the one exception discussed previously, the enzyme activity was higher in the late part of the light phase, prior to the onset of the dark phase. Overall these results suggest that the enzyme activity was at a minimum in the early part of the light phase rising during the later part of the light phase to a maximum in the dark phase.

The serum corticosterone followed a similar pattern of variation, with the concentrations obtained during the dark phase significantly higher compared with those obtained during the light phase, for the first three experiments ($p < 0.001$, except experiment 3 16.00 h-04.00 h $p < 0.05$). Due to the unusually low corticosterone concentrations during the dark phase in experiment 4, the differences between the concentrations in the dark and light phases were not statistically significant. As was generally found with the CEH activity, there was no significant difference between the levels of corticosterone measured during the dark phase for each experiment. The results suggested that the concentration of corticosterone in the blood was at a minimum in the early part of the light phase, rising to a peak concentration throughout the middle of the dark phase.

It was known that ACTH exhibited a diurnal variation in rats (Ixart, Szafarczyk et al (1977)). From a minimum at the beginning of the light phase, it rose throughout this phase to a maximum at the onset of the dark phase and slowly fell throughout this phase back to a minimum at the beginning of the light phase. The results obtained here (see Fig. 8.1) show that CEH activity and serum corticosterone concentration reflect this variation in ACTH secretion. The CEH activity rose, in response to the increasing ACTH concentration, from a minimum at the beginning of the light phase to a maximum at the onset of the dark phase. Although the ACTH concentration falls during the dark phase there will

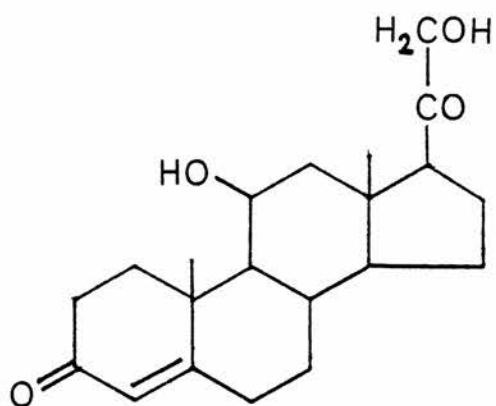
still be a concentration great enough to maintain CEH activity at a high level through much of the dark phase. The slight lack of correlation may be due to the spare ACTH receptors (McIlhinney and Schulster (1975)) with only 3% occupancy required for full steroidogenesis (Buckley and Ramachandran (1981)), and also excess cAMP is produced in stimulated cells (Podesta, Milani et al. (1979a)). However, as the ACTH concentration reaches a minimum at the onset of the light phase, the CEH activity also falls to a minimum at the beginning of the light phase. The serum corticosterone concentration reflects this variation in CEH activity and a similar pattern was observed. However there was not a sufficient number of data points in this experiment to indicate whether the corticosterone concentration lags slightly behind the CEH activity.

8.3 THE EFFECT OF CORTICOID ADMINISTRATION ON THE DIURNAL VARIATION OF CYTOSOLIC CEH ACTIVITY IN RAT ADRENAL

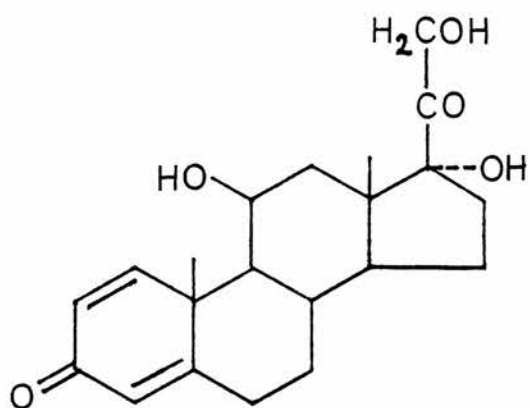
In Addison's disease, in which cortisol secretion is decreased and the plasma steroid levels are depressed, the concentration of ACTH circulating is found to be elevated (Liddle, Island and Meador (1962)). Furthermore, acute administration of cortisol produces a rapid decline in plasma ACTH concentration in man (Besser, Cullen et al. (1971)). It was concluded that the secretion of ACTH was under negative feedback regulation by the level of circulating corticoid (see James (1975)). This phenomenon is now used widely to suppress pituitary ACTH production, both in medicine (before surgery) and in biochemical research (see James (1975), McEwan (1979)). In this study two corticoid derivatives, prednisolone and dexamethasone, were used in an attempt to suppress pituitary ACTH secretion.

8.3.1 Prednisolone

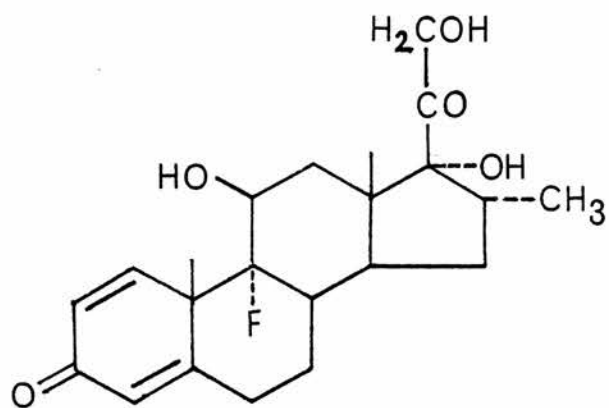
Prednisolone (1-dehydrocortisol) has the structure shown in Fig. 8.2. It is similar to the main corticoid produced by rats, corticosterone, but has an extra double bond at position 1 and a 17 α hydroxyl group.



CORTICOSTERONE
(4-Pregnen-11 β ,21-diol-3,20-dione)



PREDNISOLONE
(1,4-Pregnadien-11 β ,17 α ,
21-Triol-3,20-dione)
(1-DEHYDROCORTISOL)



DEXAMETHASONE
(1,4-Pregnadien-9-Fluoro-
16 α -methyl-11 β ,17 α ,21-Triol-
3,20-Dione)

Fig. 8.2. Structure of prednisolone, dexamethazone and corticosterone.

Rats, adapted to a 12 h light-12 h dark cycle, received subcutaneous injections of 500 μ g prednisolone in propylene glycol (200 μ l) at 72 h and 24 h before sacrifice. Control animals received 200 μ l of propylene glycol alone. The rats were killed, trunk blood and adrenals collected; serum corticosterone concentration and CEH activity in the adrenal cytosol measured, all as described in the methods section.

The results in Fig. 8.3a showed that acute prednisolone administration had only limited effect on the CEH activity in rat adrenal. At all four time points the prednisolone treated animals had lower CEH activities, but the differences were only significant at two of these points (10.00 h and 22.00 h, $p < 0.001$). Furthermore, the pattern of diurnal variation was different to the pattern observed in the previous experiments (see Fig. 8.1 and Table 8.1). In this experiment the two highest activities, which were not significantly different from each other in either the control or prednisolone-treated animals, were at the end of the light phase and the beginning of the dark phase. Although this pattern would correlate with the known pattern of ACTH secretion as well as, or better than, the pattern found previously, it was unclear why there was an apparent shift in the peak activity.

The serum corticosterone concentrations do not resolve this problem (Fig. 8.3b). The pattern observed with the control group was similar to the pattern found in Section 8.2, but the pattern observed with the prednisolone-treated group was similar to that obtained with CEH activity in this experiment. However due to the large errors and low sample numbers, none of the differences in the control group were statistically significant and only two of the differences in the prednisolone-treated group were statistically significant (10.00 h-22.00 h, $p < 0.01$ and 10.00 h-16.00 h, $p < 0.05$). Furthermore, as with the CEH activity, the differences in serum corticosterone concentration between the control group and

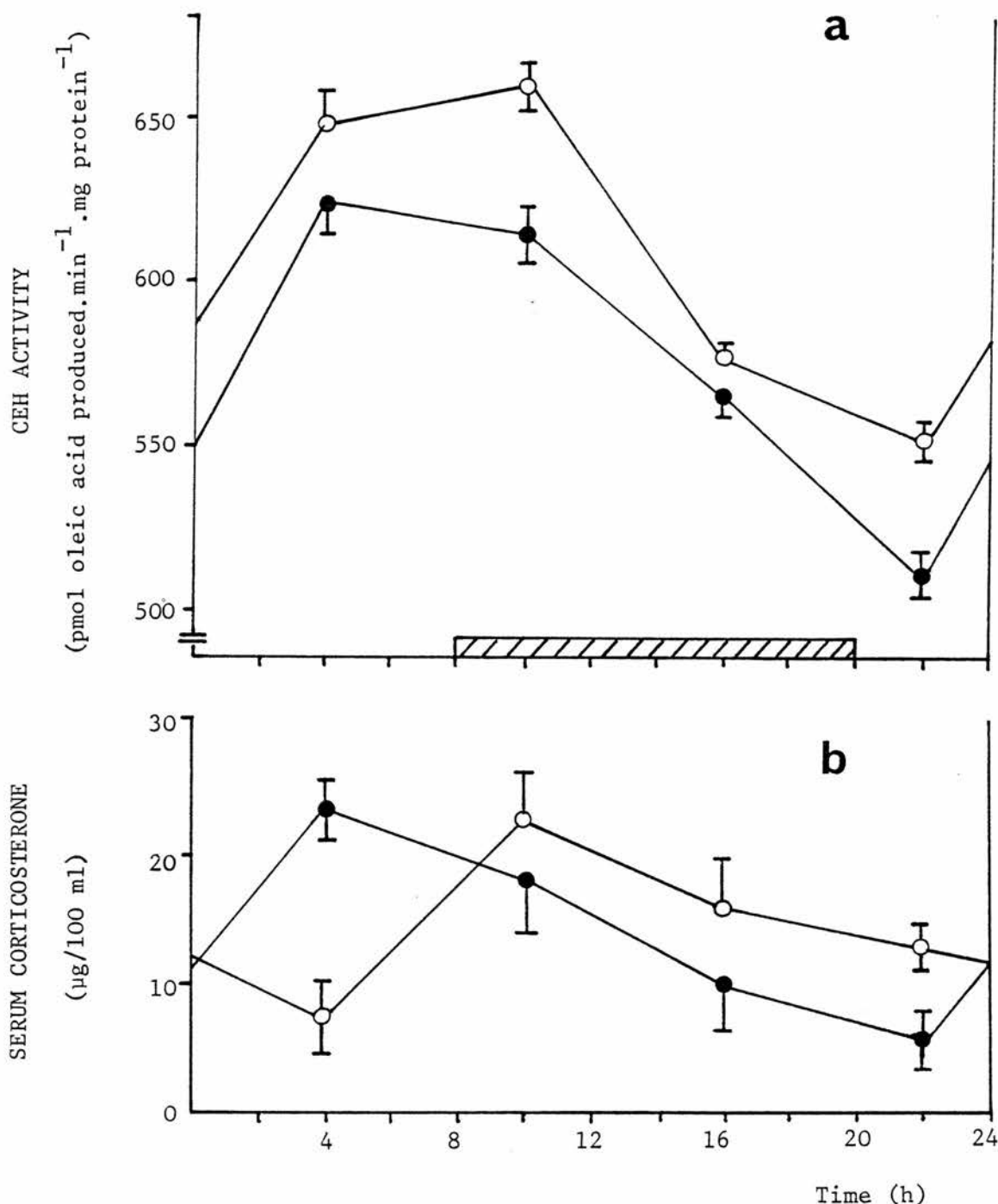


Fig. 8.3. Effect of prednisolone administration on diurnal variation of rat adrenal cytosolic CEH activity and serum corticosterone concentration. Rats were kept on a 12 h light-12 h dark cycle for 2 weeks. Half the rats were injected with prednisolone in propylene glycol, and the control animals received only propylene glycol. Details of the experiment are given in the text.

a, CEH activity
b, Serum corticosterone concentration

○, propylene glycol;
●, prednisolone.

For CEH activity the points are means \pm S.E.M. from 12 assays.
For corticosterone concentrations the points are the mean values obtained from 3 rats \pm S.E.M.

▨, Dark phase.

the prednisolone-treated group were only significant at two time points (04.00 h and 22.00 h). However, at 04.00 h the serum corticosterone concentration was higher in the prednisolone-treated group than in the control group.

These results indicated that prednisolone treatment did have some effect in lowering both CEH activity and serum corticosterone concentration, apart from the exception noted above. However, at the doses used in this experiment, the corticoid-induced suppression was not sufficient to significantly alter the pattern or degree of diurnal variation observed with CEH activity in rat adrenal cytosol.

8.3.2 Dexamethasone

The structure of dexamethasone is shown in Fig. 8.2. Similar to prednisolone it has an extra double bond at position 1 and the 17α hydroxyl group, but in addition it also contains a fluorine at position 9 and a 16α methyl group.

Rats were adapted to a 12 h light-12 h dark cycle as described in the methods section. Half the animals received subcutaneous injections of 500 μ g dexamethasone in 200 μ l saline at 72 h and 24 h before sacrifice. The control group received 200 μ l of saline alone. The animals were killed, trunk blood and adrenals collected; serum corticosterone concentration and CEH activity in the cytosol measured, all as described in the methods section.

Fig. 8.4a shows that acute dexamethasone treatment substantially reduced the CEH activity at all points in the 24 h cycle. All the activities for the control group were significantly different to each other as were all the points in the dexamethasone-treated group ($p < 0.001$). Furthermore, all the activities in the dexamethasone-treated group were significantly lower than the corresponding activities in the control group ($p < 0.001$). The differences represent a decrease in CEH activity

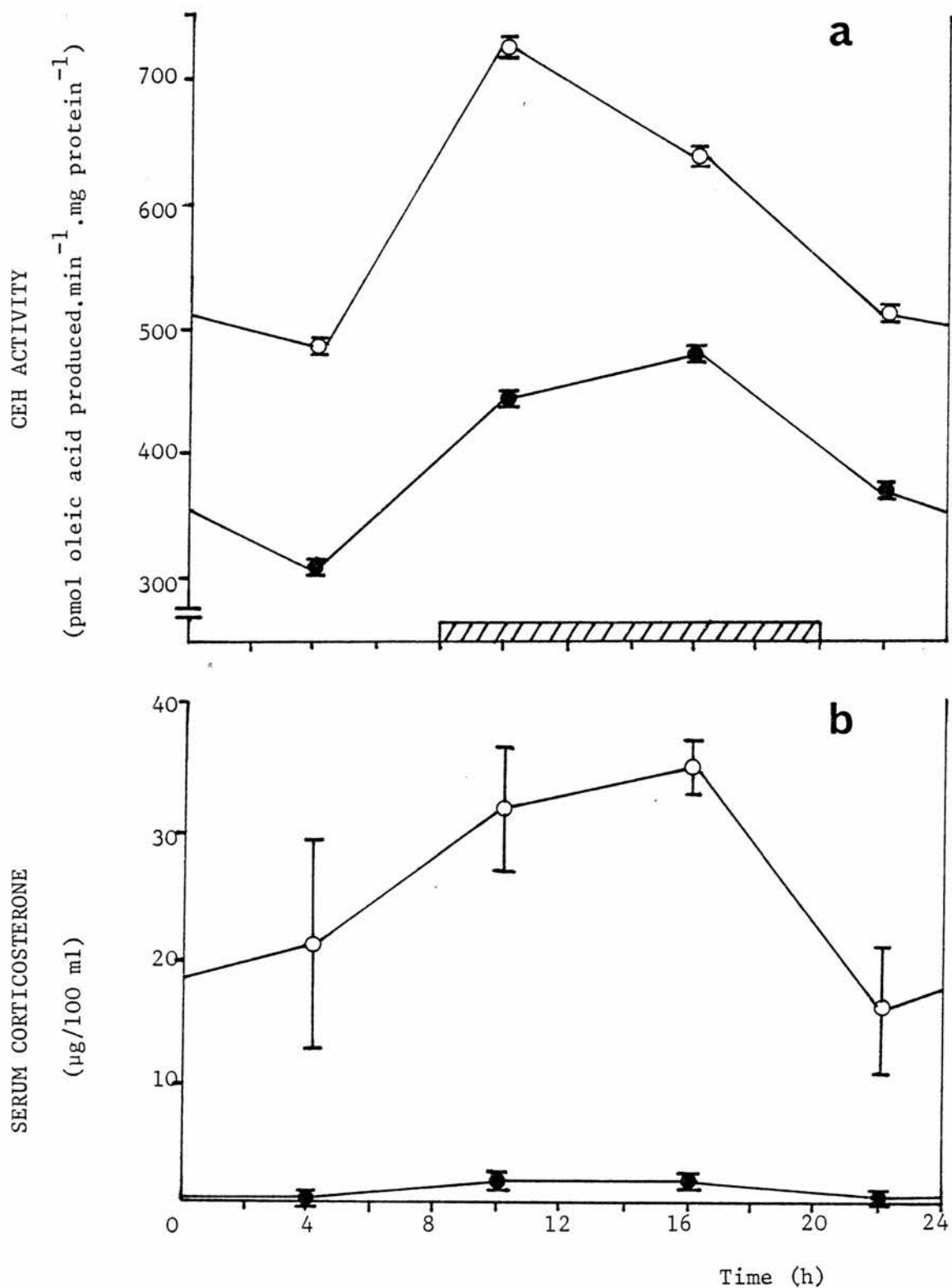


Fig. 8.4. Effect of dexamethasone administration on the diurnal variation of rat adrenal cytosolic CEH activity and serum corticosterone concentration. Rats, adapted to a 12 h dark-12 h light cycle, received subcutaneous injections of either dexamethasone in saline or saline alone. Details of the experiment are given in the text. The results are expressed as described in the legend to Fig. 8.3.

▨, Dark phase.

a, CEH activity
b, serum corticosterone concentration } O, saline;
●, dexamethasone.

of on average 31%. The pattern of diurnal variation was not altered greatly, although the peak activity was shifted from the beginning of the dark phase in the control group to later in the dark phase in the dexamethasone-treated group. Considering the variation observed in Section 8.2 in this respect, this probably does not constitute a major difference.

The degree of variation between the highest and lowest activities was 34% and 37% in the control and dexamethasone-treated groups, respectively. However as the activity was higher in the control group the variation in absolute activity was greater in the control group (253 pmol oleic acid produced.min⁻¹.mg protein⁻¹) compared to the dexamethasone-treated group (183 pmol oleic acid produced.min⁻¹.mg protein⁻¹). The results showed that dexamethasone treatment did affect CEH activity in the rat adrenal.

The serum corticosterone concentration results showed almost total suppression of corticosterone output in the dexamethasone-treated group (Fig. 8.4b). The pattern of diurnal variation was the same as observed in Section 8.2, although due to the small sample size and the large errors, none of the concentrations in either the control group or in the dexamethasone-treated group were statistically significantly different from each other. However all the values obtained in the dexamethasone group were much lower than the corresponding values in the control group, although due to the spread of results in the control group at the two time points during the light phase, the difference there was not statistically significant. It was clear that dexamethasone had had an effect, as only low levels of corticosterone were found in the treated animals. Even at these low serum corticosterone concentrations, a slight diurnal variation was observed.

Taking the CEH activity and serum corticosterone concentration results together, there appeared to be a contradiction. Although CEH activity was significantly reduced, the enzyme was still reasonably active at all time points and so the almost complete suppression of corticosterone output cannot be explained simply in terms of CEH activity. This was expected as ACTH affects the steroidogenic pathway at a number of points other than CEH. For instance ACTH affects protein synthesis and stimulates cholesterol side chain cleavage, probably via increased transport or binding of cholesterol to the cytochrome P-450_{scc} rather than an intrinsic effect on the enzyme activities (see Tait, Tait and Bell (1980)). Therefore suppression of ACTH will also affect the steroidogenic pathway at these points after the CEH step.

Furthermore the net flux of free cholesterol in adrenocortical cells will be determined by the relative activities of CEH and acylcoenzyme A:cholesterol acyl transferase (ACAT), the microsomal enzyme catalysing the formation of cholesterol ester from free cholesterol and acylcoenzyme A (see Fig. 1.1). It has been shown that stored cholesterol esters in macrophage foam cells are constantly in a cycle of hydrolysis and re-esterification (Brown, Ho and Goldstein (1980)). Net hydrolysis in these cells was obtained by a reduction in the rate of ACAT-mediated esterification and not an increase in hydrolysis. Very little information was available on the regulation of microsomal ACAT activity in the adrenal gland. However it was probable that the cholesterol esters in adrenal cortex were in a dynamic equilibrium situation, undergoing a continuous cycle of hydrolysis and re-esterification. Therefore net flux of free cholesterol could be achieved by alteration of either CEH or ACAT activity. Therefore when CEH activity was reduced in the corticoid-induced ACTH-suppressed animals, the ACAT activity may now predominate and so the net effect of the cholesterol substrate cycling would be

towards esterification. Such a situation would lead to an even greater effect on corticosteroid secretion (see Fig. 1.1).

Therefore the results obtained with dexamethasone treatment supported the theory that adrenal cytosolic CEH activity was responding in vivo to changes in secreted ACTH levels.

8.4 THE EFFECT OF ACUTE ACTH ADMINISTRATION ON THE DIURNAL VARIATION OF CYTOSOLIC CEH ACTIVITY IN RAT ADRENAL

Section 8.2 showed that cytosolic CEH activity in the rat adrenal varied diurnally in a pattern reflecting the known diurnal variation in ACTH secretion in the rat (Ixart, Szafarczyk et al. (1977)). The use of dexamethasone to suppress pituitary ACTH secretion gave a corresponding decrease in CEH activity and corticosterone secretion and supported the view that CEH varied in vivo in response to plasma ACTH levels (Section 8.3.2). In this section the effect of acute ACTH administration to rats before sacrifice was investigated, to further elucidate the relationship between ACTH and CEH in vivo.

Rats were adapted to a 12 h light-12 h dark cycle as described in the methods section. Half the animals received subcutaneous injections of ACTH in 0.2 ml saline, 30 min before sacrifice. The control group received 0.2 ml saline alone. The animals were killed, trunk blood and adrenals collected; serum corticosterone concentration and CEH activity in the cytosol measured, all as described in the methods section.

The results in Fig. 8.5a show that ACTH administration in vivo resulted in a significant increase in CEH activity at all points in the light-dark cycle. The increase was about 30% at both points during the dark phase and 13% and 68% at the early and late points in the light phase, respectively. The diurnal variation observed in the control group was not the pattern commonly obtained but was similar to a pattern obtained once before (see experiment 2, Table 8.1a). ACTH treatment

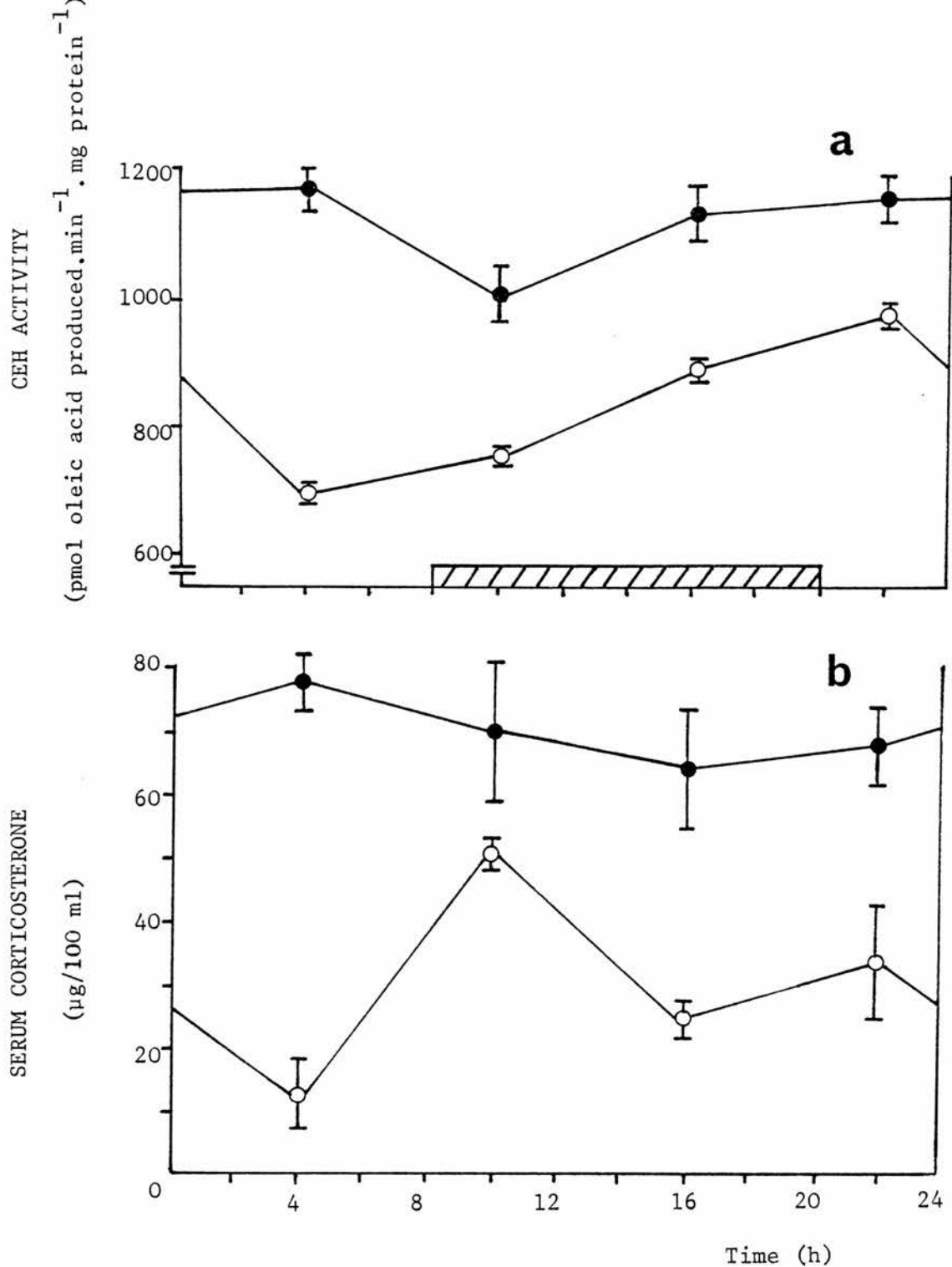


Fig. 8.5. Effect of acute ACTH administration on the diurnal variation of rat adrenal cytosolic CEH activity and serum corticosterone concentration. Rats, adapted to a 12 h dark-12 h light cycle, received subcutaneous injections of either ACTH or saline, 30 min before sacrifice. Details of the experiment are given in the text. The results are expressed as described in the legend to Fig. 8.3.

, Dark phase.

a, CEH activity
b, Serum corticosterone concentration } O, saline; ●, ACTH.

substantially reduced the variation observed and there was no significant difference between the CEH activities measured at 04.00 h, 16.00 h and 22.00 h.

ACTH treatment increased the serum corticosterone concentration at all points in the light-dark cycle, although the difference between ACTH treatment and the control at 10.00 h was not statistically significant (Fig. 8.5b). As observed with the CEH activities, the diurnal variation observed in the control group was not the pattern commonly obtained but again was similar to a pattern obtained once before (see experiment 4, Table 8.1b). ACTH treatment almost totally abolished the pattern of diurnal variation and there was no significant difference between the serum corticosterone concentrations at any of the time points after ACTH administration. These results indicated that the normal diurnal variation in rat adrenal cytosolic CEH activity and serum corticosterone could be abolished by acute ACTH administration. This supported the view that rat adrenal cytosolic CEH activity varied in vivo in response to changes in blood ACTH concentration.

8.5 SUMMARY

1. CEH activity in the rat adrenal cytosol was found to vary diurnally in a pattern reflecting the known diurnal variation of ACTH secretion.
2. The serum corticosterone concentration closely paralleled the variation in adrenal cytosolic CEH activity.
3. Prednisolone in propylene glycol was relatively ineffective, at the doses used, in suppressing either the activity of CEH or the serum corticosterone concentration.
4. Dexamethasone was effective in reducing serum corticosterone concentration to low levels, and substantially lowering CEH activity.
5. The normal pattern of diurnal variation was still observed in the corticoid suppressed animals.

6. Acute ACTH administration almost totally abolished the normal pattern of diurnal variation in both rat adrenal cytosolic CEH activity and serum corticosterone concentration.
7. The results supported the view that cytosolic CEH activity in rat adrenal varied in vivo in response to changes in ACTH concentration in the blood.

SECTION 9

INVESTIGATION OF THE APOPROTEINS OF RAT ADRENAL AND BOVINE

ADRENOCORTICAL LIPID DROPLETS

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9.1 INTRODUCTION

The substrates for CEH are long chain fatty acid esters of cholesterol. In the adrenal cortex, cholesterol esters are stored in large lipid droplets found in the cytoplasm (Garren, Gill et al. (1971)). The lipid droplets contain 70-80% of the total adrenal cholesterol and about 80-90% of this cholesterol was found to be esterified to long chain unsaturated fatty acids (Moses, Davis et al. (1969), Goodman (1965), Beckett and Boyd (1975)). It was known that when animals were subjected to stressful situations, or when ACTH was administered, there was a reduction in the cholesterol ester content of the adrenal cortex (Sayers, Sayers et al. (1944), Davis and Garren (1966)). It was found that ether anaesthesia stress, which increases the ACTH concentration in the blood, caused an increase in the activity of cytosolic CEH and depletion of lipid droplet cholesterol esters (Trzeciak and Boyd (1973)). These experiments established cholesterol esters in the lipid droplets as the substrate for cytosolic CEH in vivo.

It had been shown that dietary factors which alter the fatty acid composition in the lipid droplet cholesterol esters could have effects on the activity of cytosolic CEH (Beckett and Boyd (1975), Vahouny, Hodges and Treadwell (1979)). There have been further studies on the lipid composition of the lipid droplets in ovarian tissue (Armstrong and Flint (1973)) and rat adrenals (Mrotek, Mathew et al. (1981)) under different conditions, and on the use of rat adrenal lipid droplets as substrate for the cholesterol side chain cleavage reaction by ACTH-stimulated mitochondria (Farese, Prudente and Chuang (1980)). However, adrenal lipid droplets contain about 2% by weight of protein, but little work had been performed to investigate this protein component (Boyd and Trzeciak (1973)).

The adrenal lipid droplets have similar physical and chemical properties to the well characterised plasma lipoproteins (reviewed Scanu and Landsberger (1980), Chapman (1980), Levy (1981)). The apoproteins of the lipoproteins have been shown to be more than structural components and many have metabolic functions such as binding to cellular receptors (apolipoproteins B and E), transfer of lipid components (apolipoprotein D) and activation of lipid metabolising enzymes such as lipoprotein lipase (apolipoprotein C-II) and lecithin: cholesterol acyl transferase (apolipoprotein A-I) (see Owen and McIntyre (1982)). Considering the knowledge obtained from the study of the apoprotein moiety of plasma lipoproteins it was surprising that there had been no investigation of the protein component of adrenal lipid droplets.

The aim of this work was to investigate the protein components of isolated rat adrenal and bovine adrenocortical lipid droplets to obtain information about their nature and properties. Their relationship to cytosolic CEH was investigated and some preliminary experiments studying the effect of bovine adrenocortical lipid droplets on bovine cytosolic CEH activity were performed to determine if the apoproteins had any metabolic function, other than a structural role.

9.2 INVESTIGATION OF THE PROTEINS ASSOCIATED WITH RAT ADRENAL AND BOVINE ADRENOCORTICAL LIPID DROPLETS BY SDS/PAGE

Rat adrenal and bovine adrenocortical lipid droplets were isolated and washed as described in the methods section. Two different methods were used to delipidate the lipid droplets before investigation of the proteins by SDS/PAGE. The first method was based upon the methods of Scanu, Lewis and Bumpus (1958) devised for the delipidation of plasma lipoproteins. The protein fraction obtained by this method was termed apoACLD (apoadrenocortical lipid droplets). The second method was

devised for the rapid delipidation and preparation of the lipid droplet proteins specifically for SDS/PAGE.

9.2.1 Delipidation of lipid droplets by treatment with ethanol/ether

Lipid droplets (3 ml rat and 5 ml bovine) had 10 volumes of ice-cold ethanol/diethyl ether (1:4, v/v) added to them and were left for 2 h at 0°C. The layers were separated by the addition of $\frac{1}{5}$ volume of distilled water. The aqueous layer was re-extracted with ethanol/diethyl ether (1:4, v/v) and the re-extracted aqueous layer was lyophilised. The freeze-dried apoACLD fractions ~~were~~ dissolved in a small volume of 6 M urea/1 mM EDTA and dialysed against 5 mM potassium phosphate buffer pH 7.4 overnight to remove the urea. Samples of the apoACLD solutions were prepared and subjected to SDS/PAGE as described in the methods section.

Fig. 9.1 shows that little protein was present in the apoACLD preparations. However a similar protein pattern was observed in rat and bovine apoACLD. The major protein had Mr about 40 000 and there were other proteins with Mr 65 000, 53 000 and 12 000. Although these proteins had molecular weights similar to some of the standards used they were not due to cross-contamination as bands corresponding to the other molecular weight markers used (125 000, 92 000, 23 500) were not observed. The Mr 65 000 and 53 000 proteins were more prominent in bovine apoACLD, whereas the Mr 12 000 protein was more prominent in rat apoACLD. These results suggested that 4 major proteins or protein subunits were associated with adrenal lipid droplets and that the proteins were similar in rat and bovine tissue.

9.2.2 Delipidation of lipid droplets by treatment with acetone

The method described in Section 9.2.1 was long and tedious. Furthermore in future experiments (see Section 9.3) whole, non-delipidated lipid droplets were labelled with ^{32}P using $\left[\gamma\text{-}^{32}\text{P}\right]\text{ATP}$. It would be impractical to use that delipidation method, with a lyophilisation step,

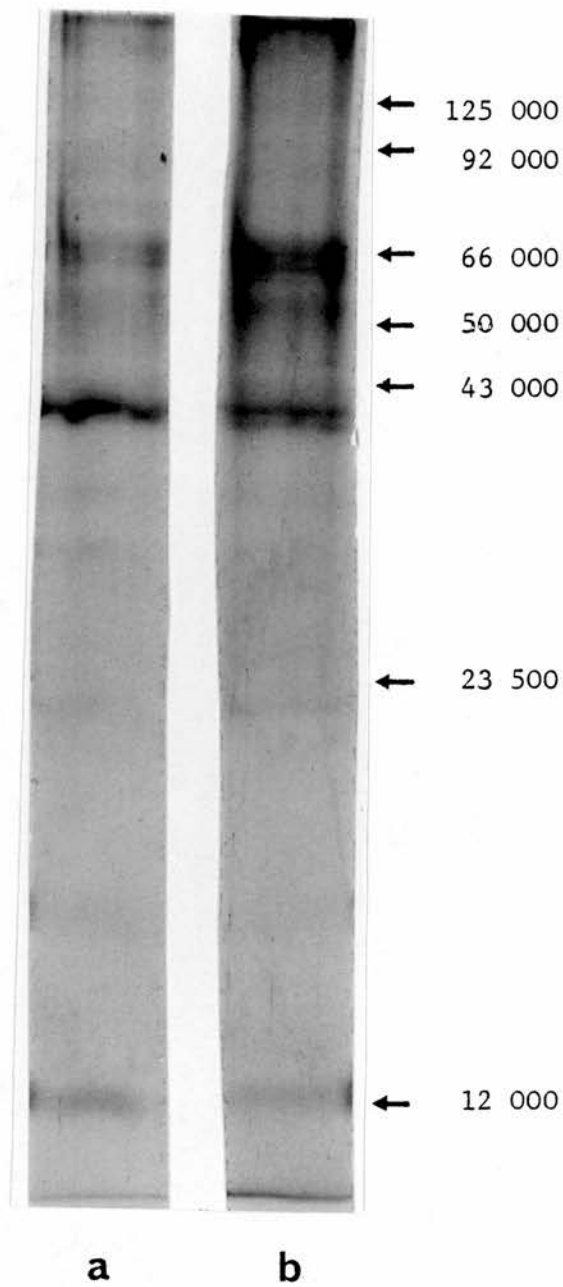


Fig. 9.1. SDS/PAGE of rat and bovine apoACLD:delipidated by treatment with ethanol:diethyl ether. Rat adrenal and bovine adrenocortical lipid droplets were delipidated by ethanol:ether extraction as described in the text. Samples of the apoACLD's (corresponding to approximately 2 ml lipid droplets) were prepared and subjected to SDS/PAGE on a 10% gel and stained for protein as described in the methods section. a, Rat apoACLD; b, Bovine apoACLD. The arrows indicate the positions on the gel of molecular weight standards.

when handling material labelled with ^{32}P . Therefore it was decided to use a modification of the method used for the preparation of protein samples for SDS/PAGE (see methods section) in these experiments.

The method adopted is described below.

Washed rat and bovine lipid droplets had ice-cold 50% (w/v) TCA added to them up to a final concentration of 20% (w/v) TCA. The samples were mixed and left for 10 min at 0°C , before centrifugation at $1000 \times g$ for 10 min. After centrifugation there was, occasionally, some precipitated material but most of the lipid droplets were found in a distinct, floating layer. The infranatant was carefully poured away leaving the lipid droplet layer attached to the side of the tube. The concentrated lipid droplets were delipidated by the addition of 5 ml ice-cold acetone. After mixing, the tubes were centrifuged at $1000 \times g$ for 5 min and the acetone carefully removed by aspiration. The protein pellet was washed with a further 2 ml ice-cold acetone, and then dried by vacuum desiccation. The protein pellets were dissolved in 50 μl 8 M urea/10% (w/v) SDS and 15 μl dissociation buffer (see methods section), boiled for 5 min and subjected to SDS/PAGE as described in the methods section.

Fig. 9.2 shows that this method of delipidation had a greatly improved recovery over the first method. The results show that the Mr 40 000 protein or subunit was the major protein component of adrenal lipid droplets in both rat and bovine tissue. However there were many other proteins associated with the lipid droplets. With bovine lipid droplets further protein bands were observed at Mr >200 000, 140 000, 90 000, 75 000, 53 000, 47 000, 31 000, 28 000, 21 000, 20 000, 14 000 and 12 000. Most of these proteins were also observed with rat lipid droplets, and no major differences between the two species were apparent. Of the proteins found after delipidation by ethanol/ether

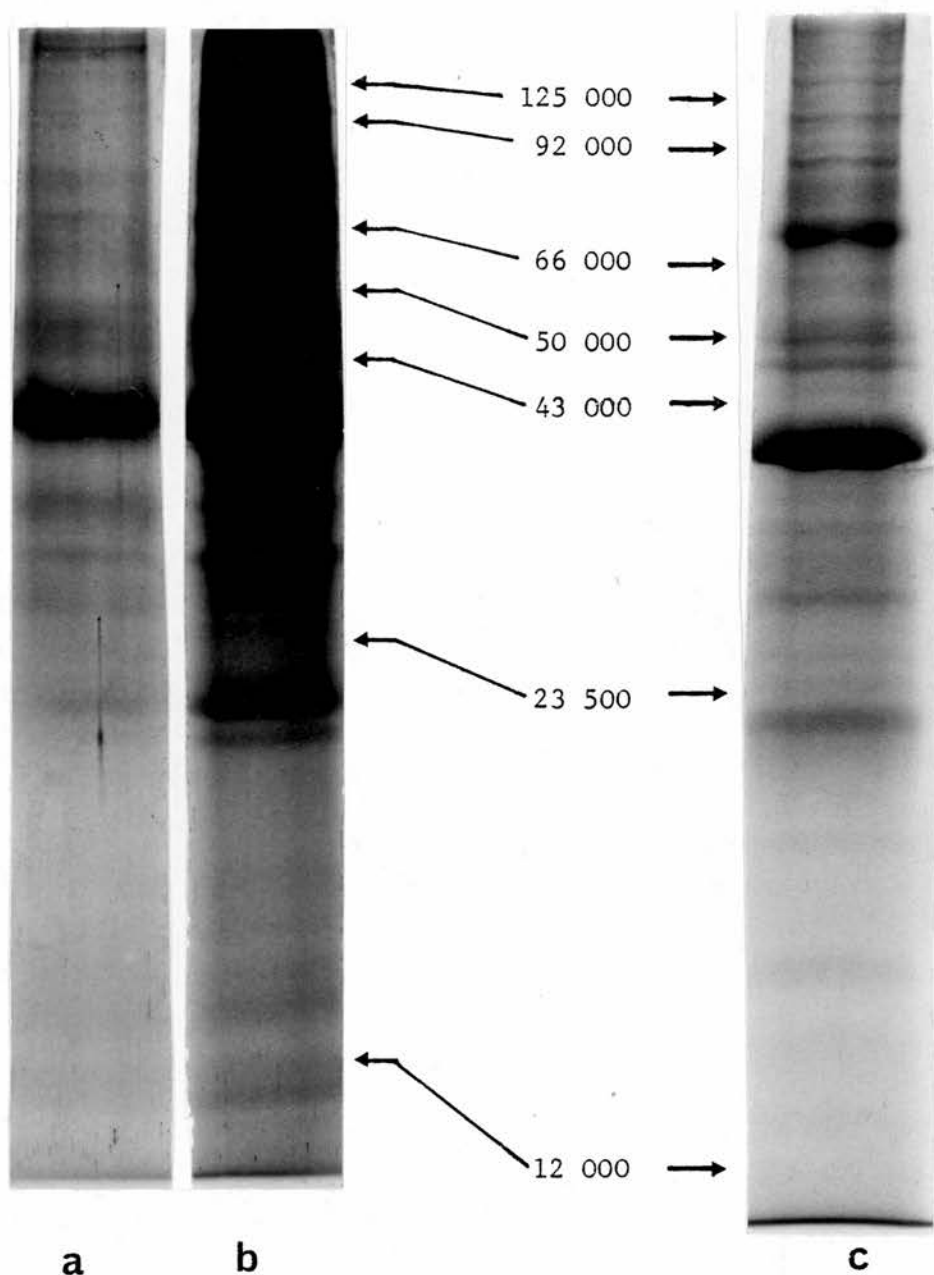


Fig. 9.2. SDS/PAGE of rat adrenal and bovine adrenocortical lipid droplet proteins:delipidated with acetone. The lipid droplets were delipidated with acetone as described in the text, and the delipidated proteins subjected to SDS/PAGE on a 10% gel and stained for protein as described in the methods section.

a, Delipidated rat lipid droplets (2 ml); b, Delipidated bovine lipid droplets (3.5 ml); c, Delipidated bovine lipid droplets (1 ml).

The arrows indicate the positions on the gel of molecular weight standards.

only the Mr 65 000 protein was now no longer apparent. With the exception of the Mr 40 000 protein, none of the other proteins were major proteins.

These results show that both bovine and rat lipid droplets have an apoprotein with a major subunit of Mr 40 000. Several minor proteins with a wide range of molecular weights can be observed, and the pattern was essentially the same in both species. Delipidation of the lipid droplets by ethanol/ether as described in Section 9.2.1 resulted in the loss of most of the minor proteins, but the Mr 40 000 apoprotein remained along with 3 other proteins. Where the major losses occur, during this delipidation procedure, was not clear as losses will occur during the extraction and also at the resolubilisation stage. Even in 6 M urea it was not possible to resolubilise all the particulate material remaining after lyophilisation. Therefore the proteins observed in Fig. 9.1 may represent the proteins that were most readily solubilised after the delipidation procedure.

A major objective in this study of lipid droplets was to determine if CEH was one of the proteins detected on the substrate. The results presented in Section 6 suggested that CEH activity was associated with a protein of Mr 82 000-84 000. The results here show that no major protein with that molecular weight was detected in the lipid droplets. This could be due to the low concentration of CEH in adrenal cytosol. Even in enzymically active preparations it was not possible to detect the CEH enzyme protein in the 150-fold purified CEH_{Q2} preparation when the gels were stained for protein.

9.3 ³²P-LABELLING OF RAT ADRENAL AND BOVINE ADRENOCORTICAL LIPID DROPLET PROTEINS USING $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

Labelling of the CEH_{Q2} preparation with ³²P using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was used, with some success, to identify the CEH enzyme protein

(see Section 6.3). This method was now used in an attempt to label the proteins associated with the lipid droplets to determine if CEH was one of the components of the protein fraction. In these experiments, both apoACLD and whole non-delipidated lipid droplets were used.

9.3.1 ApoACLD

The apoACLD fractions from rat adrenal and bovine adrenocortical lipid droplets were prepared as described in Section 9.2.1. The apoACLD in 5 mM potassium phosphate buffer pH 7.4 were incubated with $\left[\gamma\text{-}^{32}\text{P} \right] \text{ATP}$ and Mg^{2+} in the presence of the catalytic subunit of cAMP-dependent protein kinase as described in the legend to Fig. 9.3. The phosphorylated proteins were prepared and subjected to SDS/PAGE and autoradiography as described in the methods section.

With rat apoACLD there were five phosphorylated proteins with Mr 94 000, 65 000, 53 000, 40 000 and 34 000 but with bovine apoACLD only the proteins with Mr 65 000, 53 000 and 40 000 were phosphorylated (Fig. 9.3). In both cases the Mr 40 000 protein was the major phosphorylated protein, and in general the phosphorylation pattern reflected the pattern observed when the gels were stained for protein (Fig. 9.1). However the Mr 94 000 and 34 000 proteins were not detected by protein staining and the Mr 12 000 protein observed in Fig. 9.1 was not phosphorylated. The fact that the Mr 65 000 and 53 000 proteins were phosphorylated confirmed that these proteins were not due to cross-contamination from the molecular weight standards. Fig. 9.3 also shows that there was a substantial amount of material remaining on top of the stacking and separating gels suggesting that solubilisation of the material in the dissociation buffer was incomplete.

9.3.2 Whole lipid droplets

The process of delipidation with ethanol/ether, lyophilisation and solubilisation in urea may have altered the structure of the apoproteins,

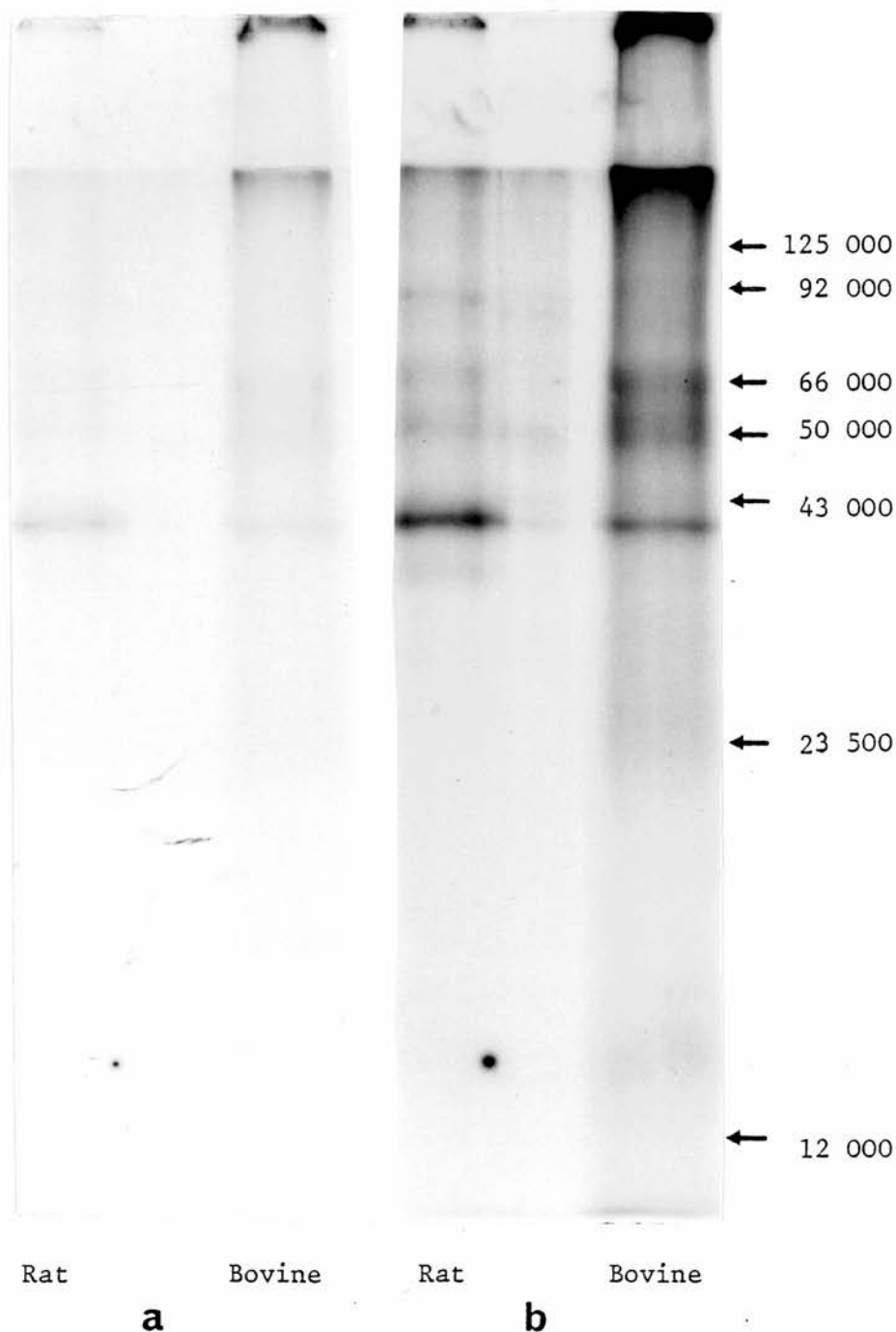


Fig. 9.3. ^{32}P -labelling of rat and bovine apoACLD. Rat (3 ml) and bovine (5 ml) apoACLD were incubated for 30 min at 37°C with 0.1 mM $[\gamma\text{-}^{32}\text{P}]$ ATP (50 μCi , 1100 cpm.pmol $^{-1}$), 0.5 mM magnesium chloride and the catalytic subunit of cAMP-dependent protein kinase (100 units). The reaction was terminated by the addition of ice-cold 50% (w/v) TCA and the precipitated proteins subjected to SDS/PAGE on a 10% gel and autoradiography as described in the methods section. Autoradiograph after a, 18 h exposure and b, 18 h exposure with presensitised film and intensifying screen.

The arrows indicate the positions on the gel of molecular weight standards. The gel stained for protein is shown in Fig. 9.1.

possibly resulting in some unfolding of the tertiary structure. This may have led to the exposure of possible phosphorylation sites not normally accessible to cAMP-dependent protein kinase in vivo.

Furthermore that method of delipidation led to great losses in the minor components of the protein fraction (Figs. 9.1 and 9.2). For these reasons it was decided to label whole, non-delipidated lipid droplets.

Rat adrenal and bovine adrenocortical lipid droplets were incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP as described in the legend to Fig. 9.3 and the phosphorylated proteins subjected to SDS/PAGE and autoradiography as described in the methods section. A major difference between rat and bovine lipid droplets was discovered in this experiment. Whereas the major apoprotein of Mr 40 000 was phosphorylated in rat lipid droplets it was not phosphorylated in bovine lipid droplets (Fig. 9.4).

This suggests that in bovine lipid droplets the cAMP-dependent protein kinase phosphorylation site(s) in the Mr 40 000 apoprotein were not accessible to the catalytic subunit. It was possible that the protein had a slightly different tertiary structure in whole lipid droplets, or that the site(s) were blocked by another closely associated protein or subunit, or by lipids. Another explanation may be that the protein was already phosphorylated when the lipid droplets were isolated. If the former explanation for the non-phosphorylation is correct, the results in Fig. 9.3 show that when the proteins were delipidated the phosphorylation site(s) were no longer hidden in the tertiary structure or blocked, and the protein was phosphorylated.

Other phosphorylated proteins in the rat lipid droplets had Mr 53 000 and 37 000 with a few minor phosphorylated proteins with Mr 26 000, 15 000 and 14 000. In bovine lipid droplets the proteins with Mr 53 000 and 37 000 were phosphorylated, although the Mr 37 000 protein was displaced due to the large amount of unlabelled Mr 40 000

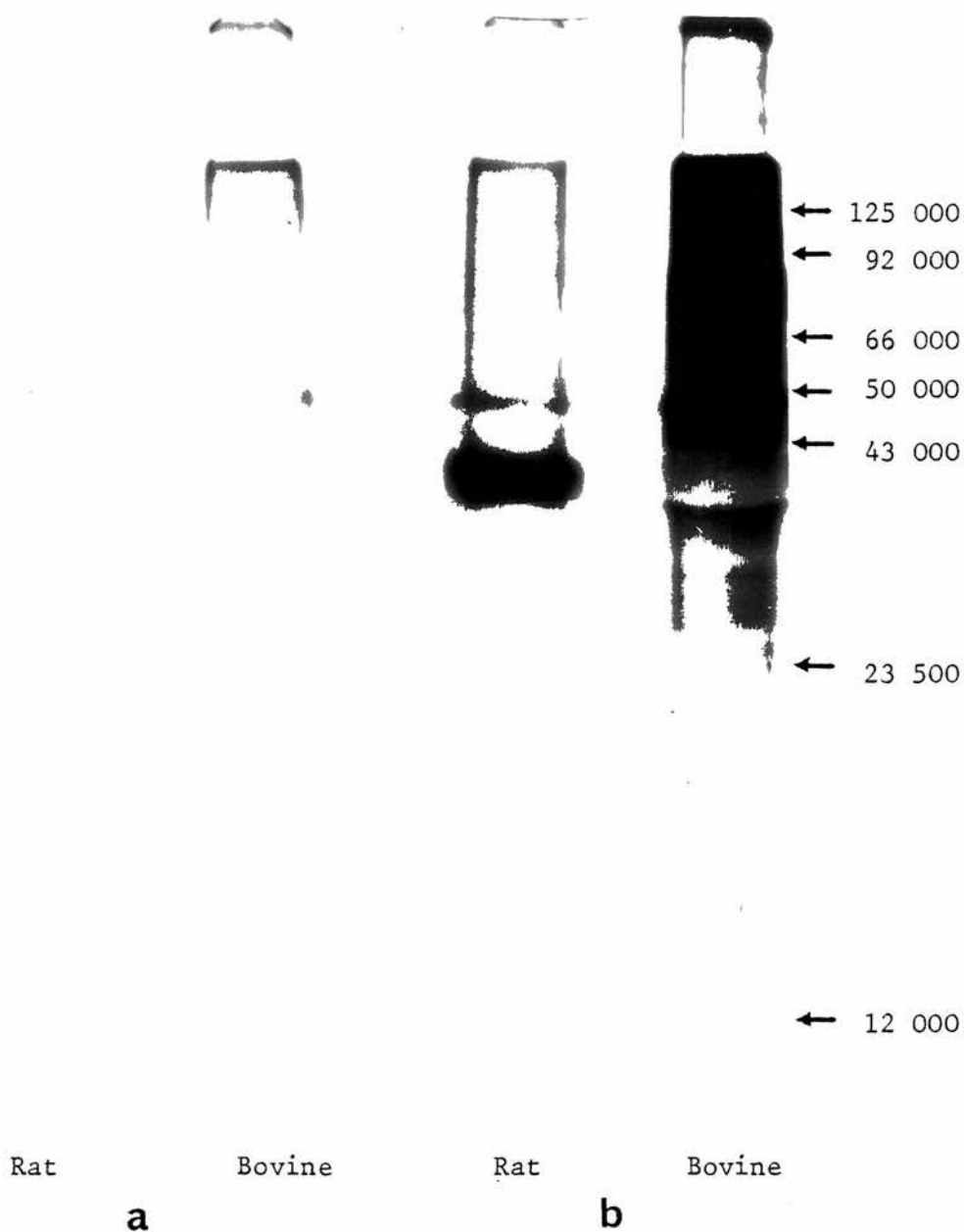


Fig. 9.4. ^{32}P -labelling of whole, non-delipidated rat and bovine lipid droplets. Rat adrenal (2 ml) and bovine adrenocortical (3.5 ml) lipid droplets were incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP as described in the legend to Fig. 9.3. The reaction was terminated by the addition of ice-cold 50% (w/v) TCA and the proteins subjected to SDS/PAGE on a 10% gel (see Section 9.2.2) and autoradiography. Autoradiograph after a, 18 h exposure and b, 18 h exposure with presensitised film and intensifying screen.

The arrows indicate the positions on the gel of molecular weight markers. The gel stained for protein is shown in Fig. 9.2.

apoprotein (see Fig. 9.2). There were several other minor phosphorylated proteins with molecular weights in the 100 000-60 000 and 20 000-12 000 range. In both cases there was some material that did not enter the gel.

No major protein with Mr 82 000-84 000 was phosphorylated in rat lipid droplets, but there was some phosphorylation at this region in bovine lipid droplets although it was a minor component. Therefore the results suggest that CEH was not a major protein associated with lipid droplets, when isolated as described in the methods section.

9.4 [1,3-³H]DFP-LABELLING OF BOVINE ADRENOCORTICAL LIPID DROPLET PROTEINS

Labelling of the partially purified CEH_{Q2} preparation with [1,3-³H] DFP had provided evidence for the identification of the CEH enzyme protein (see Section 6.4). This method was now used to further investigate the proteins associated with bovine adrenocortical lipid droplets. As in the ³²P-labelling studies, both apoACLD and whole non-delipidated lipid droplets were studied.

9.4.1 ApoACLD

Bovine apoACLD were incubated with 30 μM [1,3-³H] DFP as described in the legend to Fig. 9.5. The labelled material was split into two portions and subjected to SDS/PAGE as described in the methods section. ³H-incorporation was determined by fluorography/autoradiography or by liquid scintillation counting after elution of the protein from the excised gel slices by NCS tissue solubiliser (see methods section).

On this occasion the Mr 40 000 apoprotein was hardly visible on the gel after staining for protein and soaking in 1 M sodium salicylate (Fig. 9.5a). The Mr 65 000 protein was the only major band with minor bands at about Mr 53 000 and 20 000. No labelling of protein by ³H was detected by fluorography (Fig. 9.5b).

Determination of the ³H incorporation after gel slicing and NCS treatment gave no definite results although there may have been slight

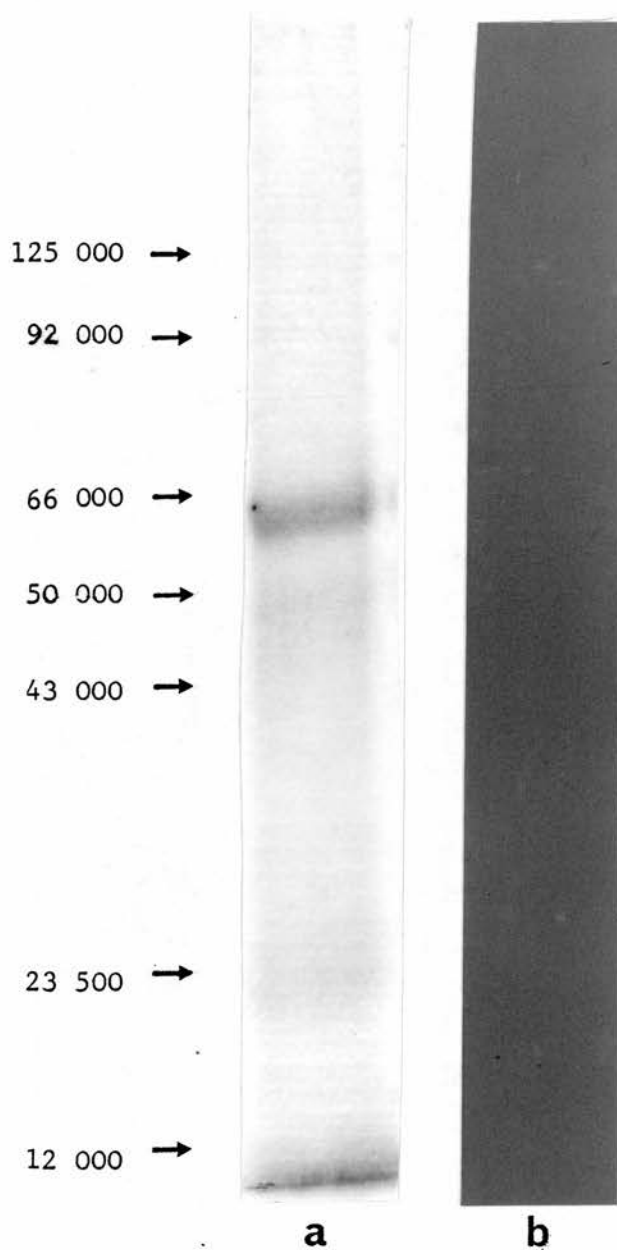


Fig. 9.5. $[1,3-^3\text{H}]$ DFP-labelling of bovine apoACLD; Fluorography. Bovine apoACLD (100 μg protein) were incubated with 30 μM $[1,3-^3\text{H}]$ DFP (6 Ci.mmol $^{-1}$) for 30 min at 37°C. The reaction was terminated by the addition of an equal volume of ice-cold 25% (w/v) TCA. The precipitated protein was subjected to SDS/PAGE on a 6-12% exponential gradient gel and fluorography as described in the methods section. a, Protein stain; b, Autoradiograph/Fluorograph. The arrows indicate the position on the gel of molecular weight standards.

incorporation into the Mr 65 000 protein and a low molecular weight protein (Mr 13 000), but the peaks at Mr 47 000 and 40 000 consisted of only one point and so were not significant (Fig. 9.6).

9.4.2 Whole lipid droplets

Agents such as DFP, label proteins at 'active-site' or 'essential' serines where the reactivity of the serine residue is increased due to its environment in the tertiary structure of the protein molecule (Sigman and Mooser (1975)).

As noted in Section 9.3.2, the delipidation procedure may alter the tertiary structure of the protein molecules. Whereas this may lead to increased phosphorylation due to the exposure of more phosphorylation sites, it was likely to reduce the amount of DFP labelling due to a loss in reactivity of serine residues. Therefore the whole lipid droplets were also incubated with $[1,3-^3\text{H}]$ DFP as described in the legend to Fig. 9.7.

Fig. 9.7a shows that the Mr 40 000 protein was the major apoprotein in this preparation with minor proteins of Mr 90 000, 68 000, 53 000/47 000, and 20 000. However the autoradiograph obtained from this gel showed that none of the proteins were labelled by $[1,3-^3\text{H}]$ DFP, even when these proteins were in a conformation much closer, presumably, to their native state (Fig. 9.7b). The results obtained when ^3H incorporation was determined after gel slicing and NCS solubilisation indicated that possibly one or two proteins with Mr about 21 000 and 20 000 were labelled (Fig. 9.8). In neither of the $[1,3-^3\text{H}]$ DFP-labelling experiments was a protein with Mr 82 000-84 000 labelled.

Overall the results obtained in Sections 9.2, 9.3 and 9.4 showed that both rat adrenal and bovine adrenocortical lipid droplets contained a major apoprotein with a subunit Mr 40 000. Several minor proteins were also detected and the pattern was similar in both species.

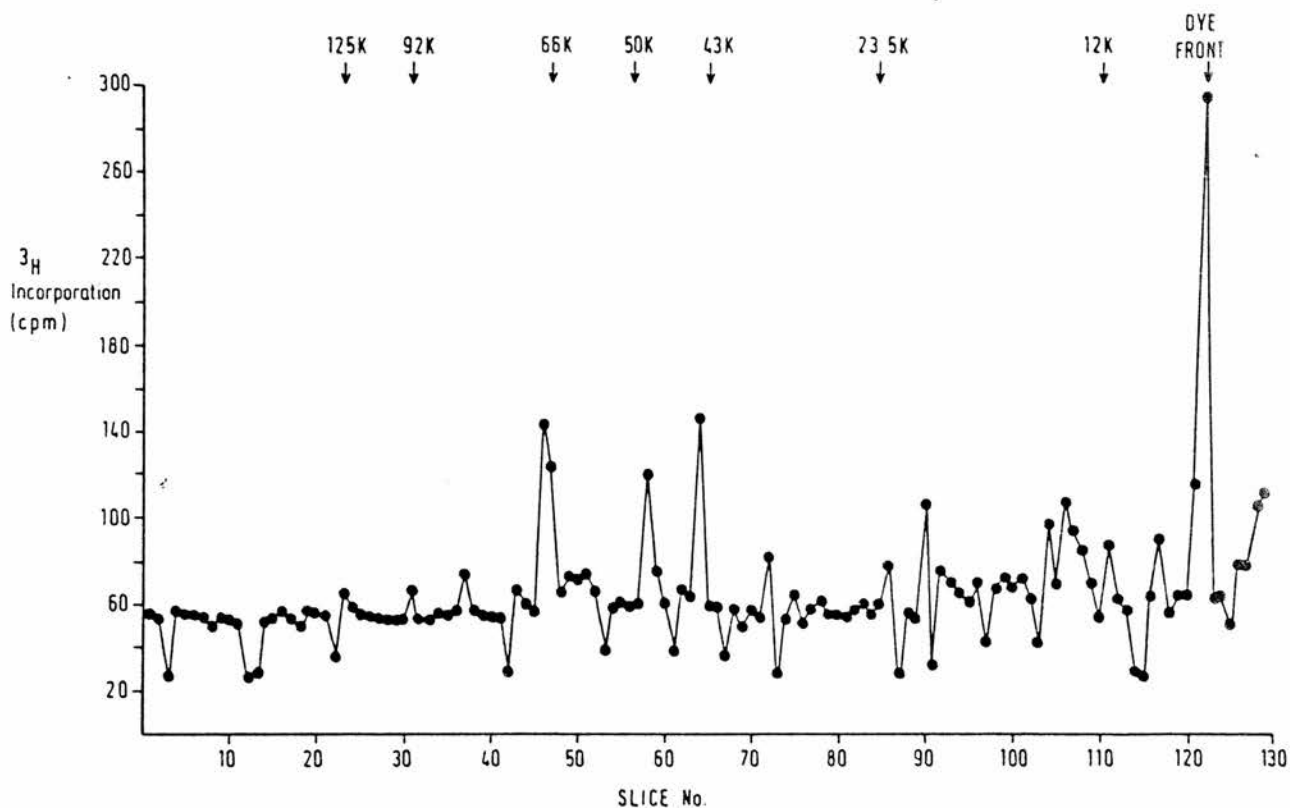


Fig. 9.6. $[1,3-^3\text{H}]$ DFP-labelling of bovine apoACLD; Gel slices.

The experiment was performed as described in the legend to Fig. 9.5 except that ^3H incorporation was determined by liquid scintillation counting after elution of the protein from excised gel slices by NCS tissue solubiliser (see methods section).

The arrows indicate the position on the gel of molecular weight standards.

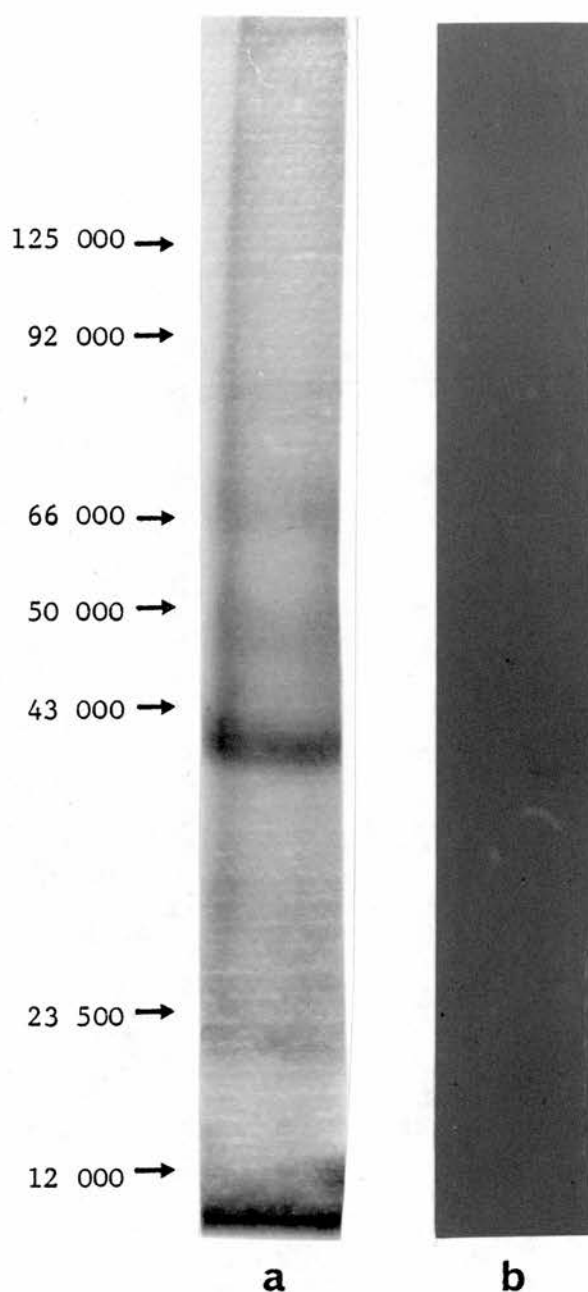


Fig. 9.7. $[1,3-^3\text{H}]$ DFP-labelling of whole bovine lipid droplets; Fluorography. Bovine lipid droplets were incubated with $30\ \mu\text{M}$ $[1,3-^3\text{H}]$ DFP ($6\ \text{Ci.mmol}^{-1}$) for 30 min at 37°C . The rest of the experimental details are as described in the legend to Fig. 9.5. ^3H incorporation was determined by fluorography as described in the methods section.

a, Protein stain; b, Autoradiograph/fluorograph.

The arrows indicate the position on the gel of molecular weight standards.

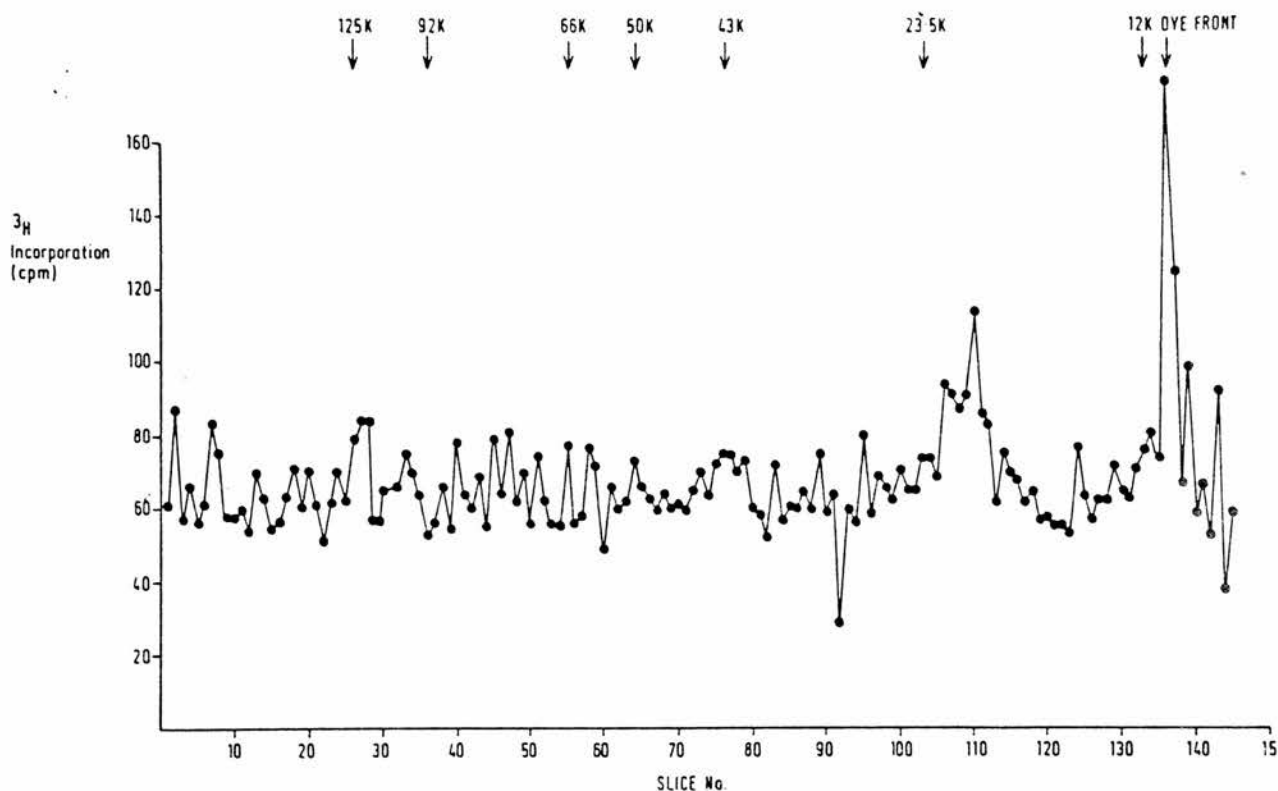


Fig. 9.8. $[1,3-^3\text{H}]$ DFP-labelling of whole bovine lipid droplets; Gel slicing. The experiment was performed as described in the legend to Fig. 9.7 except that ^3H incorporation was determined by liquid scintillation counting after elution of protein from excised gel slices by NCS tissue solubiliser (see methods section). The arrows indicate the position on the gel of molecular weight standards.

However, it was not known if the Mr 40 000 protein was identical in both species, because this protein was phosphorylated in rat lipid droplets but not in bovine lipid droplets, although it was phosphorylated in both species after the delipidation treatment. No evidence was obtained to suggest that CEH was a major component of the protein in either rat or bovine adrenal lipid droplets.

9.5 PRELIMINARY EXPERIMENTS ON THE EFFECTS OF BOVINE ADRENOCORTICAL LIPID DROPLETS ON BOVINE ADRENOCORTICAL CYTOSOLIC CEH ACTIVITY

It is well documented that some enzymes that act on hydrophobic, lipid substrates require protein cofactors for full activity. Lipoprotein lipase requires apolipoprotein C-II (Ganeson, Bradford et al.(1971)), lecithin: cholesterol acyl transferase (LCAT) requires apolipoprotein A-I (Fielding, Shore and Fielding (1972)) and pancreatic lipase requires its colipase (Borgstrom, Erlanson-Albertsson and Wieloch (1979)) for maximal enzymic activity. The substrates for these enzymes are triacylglycerols in chylomicrons and VLDL, lecithin and cholesterol in HDL and triacylglycerols complexed with bile salts and phospholipids, respectively. These substrates are similar in nature to the cholesteryl ester-rich lipid droplets that are the substrates for CEH in the adrenal cortex. It was possible that a protein associated with the lipid droplets might serve as a cofactor for CEH activity in the same way as apolipoprotein A-I in HDL and apolipoprotein C-II in chylomicrons and VLDL stimulate LCAT and lipoprotein lipase, respectively. Therefore, the effect of bovine adrenocortical lipid droplets on cytosolic CEH activity was investigated.

9.5.1 Effect of bovine adrenocortical lipid droplets on the activity of bovine adrenocortical cytosolic CEH

Bovine adrenocortical lipid droplets, prepared and washed as described in the methods section, were added in increasing amounts to a constant amount of bovine adrenocortical cytosol and the CEH activity measured as described in the methods section.

Bovine lipid droplets were found to contain CEH activity (Fig. 9.9). On this occasion, with equal volumes of these particular preparations, the CEH activity associated with the lipid droplets was about 44% of the activity in the cytosol. The activity was expressed as cpm to allow easy comparison of the two activities. The specific activity of the enzyme in the cytosol was $650 \text{ pmol oleic acid produced} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ whereas the specific activity in this lipid droplet preparation was approximately 90 times this due to the low protein concentration of the lipid droplet preparation (0.023 mg ml^{-1}).

No stimulation of the CEH activity in the cytosol was observed upon the addition of bovine lipid droplets (Fig. 9.9). The slope of the line obtained with cytosol plus lipid droplets was not significantly different from the slope of the line obtained with lipid droplets alone ($p > 0.1$). The results indicated that there was no protein in the lipid droplets that could act as a cofactor for cytosolic CEH. However the degree of interaction between the cytosolic CEH, the lipid droplets and the substrate emulsion was unknown. The results also indicated that the cholesterol esters in the lipid droplets did not interfere in the assay. The total cholesterol concentration of this preparation of lipid droplets was 0.55 mg ml^{-1} and so up to $12 \text{ } \mu\text{g}$ of cholesterol ester derived from the lipid droplets was added to the CEH assays in this experiment. As the amount of labelled cholesterol ester in the substrate emulsion was $33 \text{ } \mu\text{g}$ per assay, the lipid droplet cholesterol ester would represent a dilution of up to $\frac{1}{3}$. However no substrate inhibition was observed in this experiment.

9.5.2 Effect of bovine adrenocortical lipid droplets on the modulation of bovine adrenocortical cytosolic CEH

Although no evidence was obtained to suggest that lipid droplets contain a protein cofactor for cytosolic CEH it was possible that the

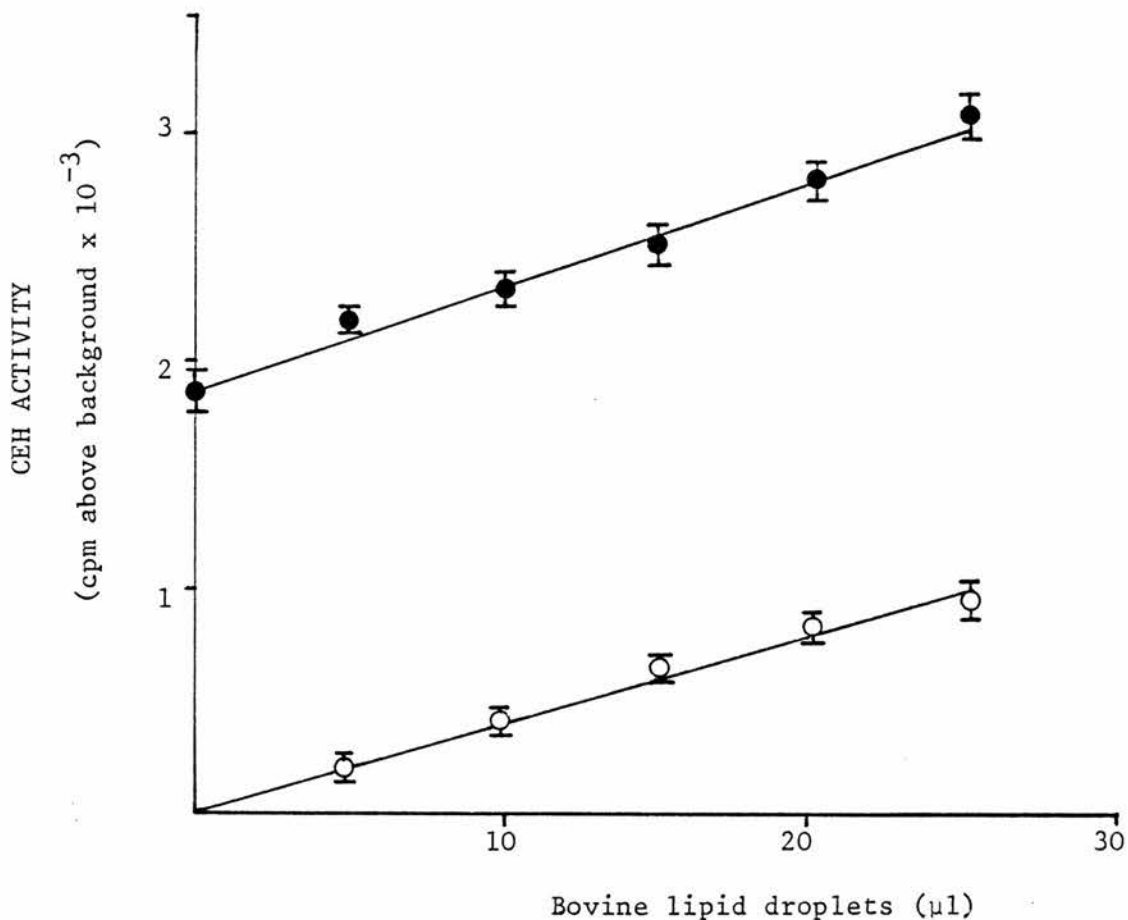


Fig. 9.9. Effect of bovine adrenocortical lipid droplets on bovine cytosolic CEH activity. Bovine adrenocortical lipid droplets (560 μg cholesterol and 23 μg protein per ml in 10 mM potassium phosphate buffer pH 7.4) were added in the amounts indicated to 20 μl bovine tissue cytosol (4.7 mgml^{-1}) followed by addition of cholesterol $[1-^{14}\text{C}]$ oleate substrate emulsion. CEH activity was determined as described in the methods section.

O, Bovine lipid droplets alone : Slope = 40.4 ± 3.0 (Standard error)

●, Cytosol plus bovine lipid droplets : Slope = 46.9 ± 3.2 (Standard error)

N.B. CEH activity was expressed as cpm above background to allow comparison. Points are means \pm S.E.M. of triplicate assays. Slopes were calculated by the method of least squares.

putative cofactor may assist or affect the modulation of CEH activity. This was investigated by preincubating bovine adrenocortical cytosol with Mg^{2+} or $Mg^{2+}/ATP/cAMP$ in the presence or absence of bovine lipid droplets as described in the legend to Fig. 9.10. Cholesterol [$1-^{14}C$] oleate substrate emulsion was then added and CEH activity determined as described in the methods section.

On this occasion the CEH activity associated with the lipid droplets was much lower and represented less than 3% of the cytosolic CEH activity (Fig. 9.10). In repeat experiments (see also Table 9.1) the CEH activity associated with lipid droplets was always of this order. Incubation of the lipid droplets with Mg^{2+} resulted in almost complete deactivation of the associated CEH activity. This suggested that there was phosphoprotein phosphatase activity associated with the lipid droplets. However incubation of the lipid droplets with $Mg^{2+}/ATP/cAMP$ did not result in activation of the associated CEH activity and so the lipid droplets did not contain cAMP-dependent protein kinase. Furthermore, no significant difference in the cytosolic CEH activity in either basal, deactivated or activated states was found upon the addition of bovine lipid droplets to the preincubations. Therefore, apart from the associated phosphoprotein phosphatase activity, there was no evidence to suggest that the protein fraction of lipid droplets could significantly affect the modulation of cytosolic CEH activity.

9.5.3 The CEH activity associated with bovine adrenocortical lipid droplets

It was clear from these preliminary experiments that there was CEH activity associated with bovine adrenocortical lipid droplets. However this activity was variable as more activity was associated with the lipid droplet preparation used in Section 9.5.1 compared to the lipid droplet preparation used in Section 9.5.2. Repeat experiments showed

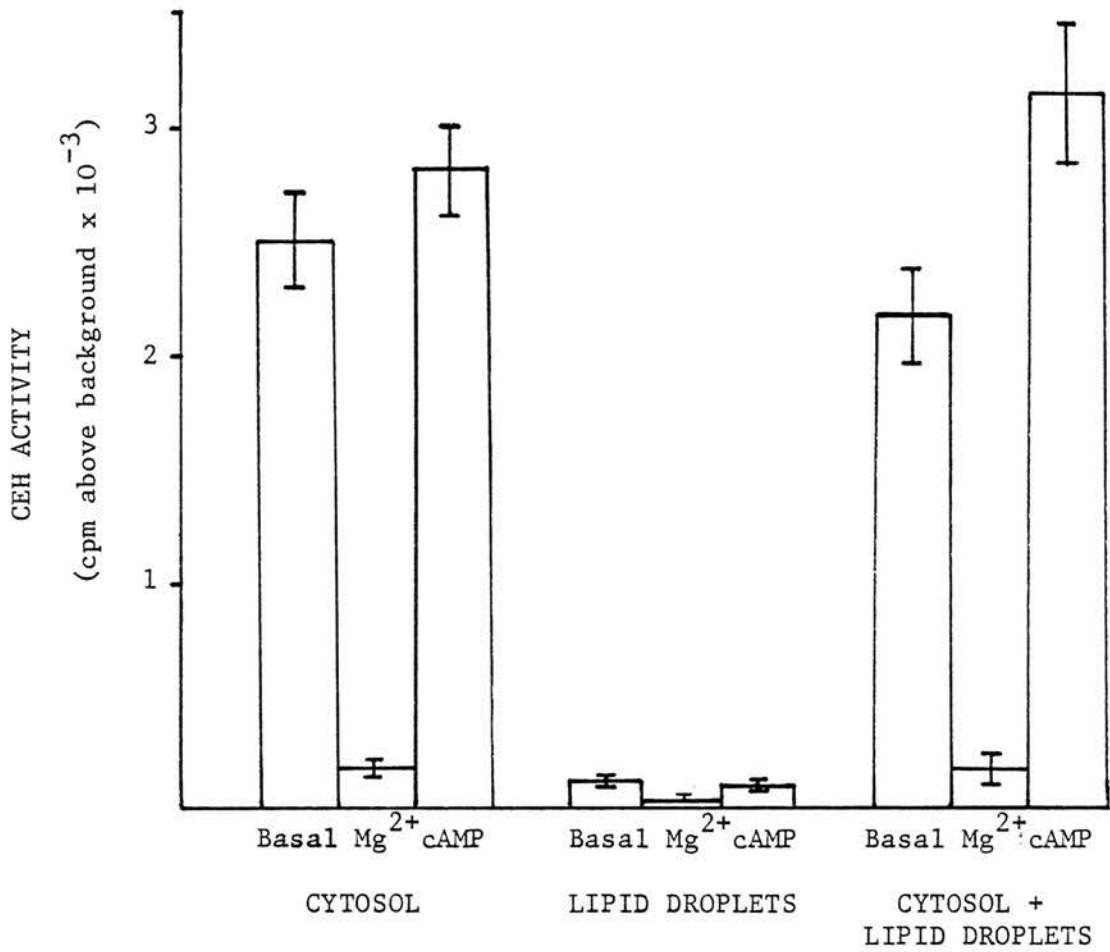


Fig. 9.10. Effect of bovine adrenocortical lipid droplets on the modulation of bovine adrenocortical cytosolic CEH activity.

Samples of cytosol (7.3 mgml^{-1}), or lipid droplets ($296 \text{ } \mu\text{g cholesterol.ml}^{-1}$), or cytosol plus lipid droplets were preincubated with basal, deactivation (Mg^{2+}) or activation (cAMP) buffers as described in the methods section. Cholesterol [$1\text{-}^{14}\text{C}$]oleate substrate emulsion was then added and CEH activity measured as described in the methods section. Results are means \pm S.E.M. of triplicate assays.

that the lower activity was more frequently observed. The reason for the higher activity obtained in Section 9.5.1 was unknown but it may be due to incomplete washing.

Furthermore, some phosphoprotein phosphatase activity was associated with the lipid droplets and the studies in Section 9.2 showed that there were minor proteins associated with lipid droplets. These results suggest that a carefully controlled and efficient washing scheme for the lipid droplets must be used, in order to remove proteins that are not integral but are merely associated with lipid droplets due to their hydrophobic nature. An experiment was performed to investigate the effect of sequential washing on the lipid droplets by monitoring total cholesterol, protein (concentration and SDS/PAGE) and CEH activity.

Table 9.1 shows the effect of sequential washing of bovine lipid droplets. The total cholesterol:protein ratio rose from 1.3 in the unwashed state to 26.3 after 2 washes. This ratio was not appreciably altered by further washing. The specific activity of CEH associated with the lipid droplets also remained constant through washes 2-4, suggesting a small amount of CEH enzyme protein was a permanent constituent of the lipid droplets. Fig. 9.11 shows that the protein patterns on SDS/PAGE were almost identical after 2 and 4 washes, and were similar to the previous results, although the problems of inconsistent solubilisation and recovery of all the proteins were again observed. However the results show that washing the lipid droplets twice (as was the case in all experiments in this study) was effective in removing loosely associated protein, and that fully washed bovine adrenocortical lipid droplets consist of about 4% protein by weight.

9.6 SUMMARY

1. Rat adrenal and bovine adrenocortical lipid droplets were found to contain a major apoprotein with a subunit of Mr 40 000.

Treatment	Protein Concentration (mgml ⁻¹)	Total Cholesterol Concentration (mgml ⁻¹)	$\frac{\text{Cholesterol}}{\text{Protein}}$	CEH Activity	
				cpm	pmol oleic acid produced. min ⁻¹ .mg protein ⁻¹
Unwashed	0.256	0.33	1.3	1498 \pm 145	5450 \pm 528
Wash 1	0.029	0.12	4.2	99 \pm 13	3197 \pm 418
Wash 2	0.008	0.21	26.3	73 \pm 11	8382 \pm 1280
Wash 3	0.006	0.17	28.3	54 \pm 9	8382 \pm 1397
Wash 4	0.005	0.13	26.0	41 \pm 4	7337 \pm 745

Table 9.1. Effect of washing on protein concentration, total cholesterol concentration and the CEH activity of bovine adrenocortical lipid droplets. Lipid droplets were prepared and washed sequentially according to the procedures described in the methods section. Results for CEH activity are means \pm S.E.M. of four assays.

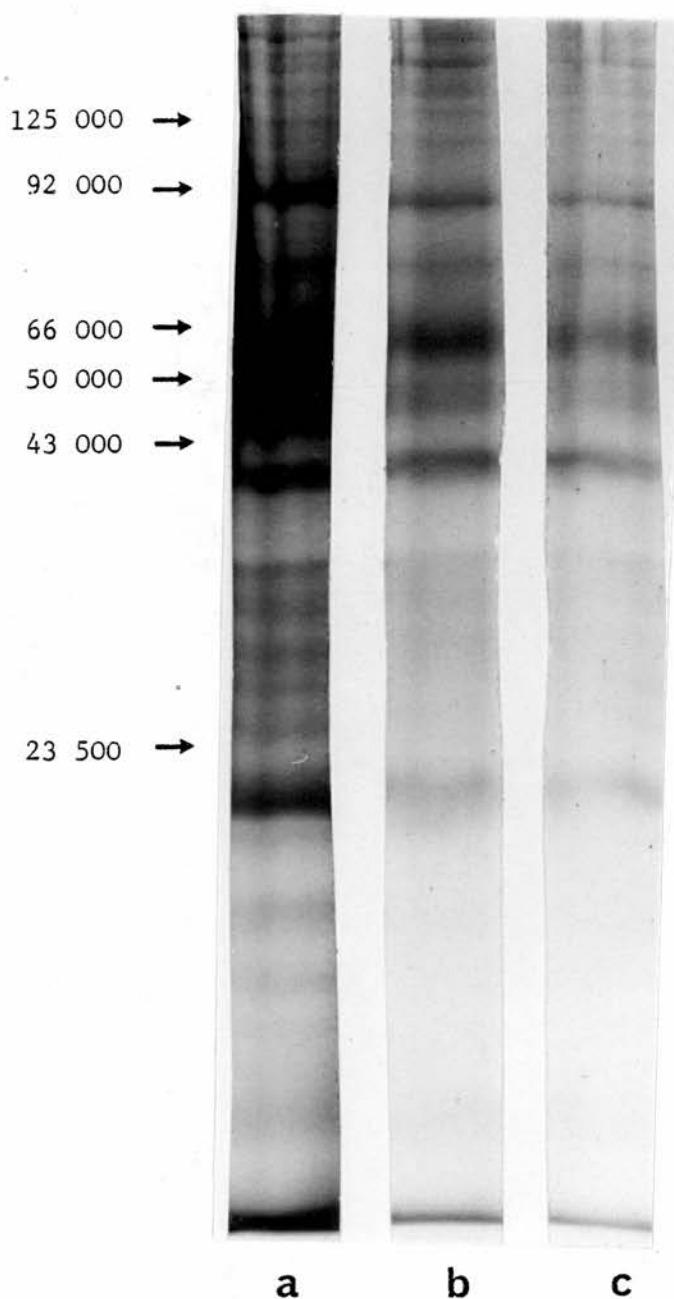


Fig. 9.11. Effect of washing on the proteins associated with bovine adrenocortical lipid droplets. Lipid droplets were prepared and washed sequentially as described in the text. Samples from different stages were subjected to SDS/PAGE on a 10% gel as described in Section 9.2.2.

a, Unwashed lipid droplets (approximately 80 μ g); b, Lipid droplets washed twice (approximately 32 μ g); c, Lipid droplets washed four times (approximately 25 μ g).

2. This apoprotein was able to be phosphorylated in rat adrenal but not bovine adrenocortical lipid droplets.
3. The delipidated apoprotein was able to be phosphorylated in both cases.
4. Various minor proteins were found in both rat adrenal and bovine adrenocortical lipid droplets, and some were able to be phosphorylated. The protein and phosphorylation patterns observed were similar in rat adrenal and bovine adrenocortical lipid droplets.
5. The major apoprotein with Mr 40 000 was not labelled by $[1,3-^3\text{H}] \text{ DFP}$ but one or two minor proteins may be labelled by this reagent.
6. No evidence was obtained to suggest that CEH was a major component of the protein fraction of rat adrenal or bovine adrenocortical lipid droplets. However there was some CEH activity associated with bovine adrenocortical lipid droplets.
7. Phosphoprotein phosphatase may also be associated with bovine adrenocortical lipid droplets, but cAMP-dependent protein kinase was not detected.
8. Sequential washing showed that bovine adrenocortical lipid droplets contained 4% by weight of protein.
9. There was no evidence to suggest that any protein in bovine adrenocortical lipid droplets could act as a cofactor for cytosolic CEH activity.

SECTION 10

GENERAL DISCUSSION

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10.1 GENERAL DISCUSSION

The primary aim in the study of adrenocortical cytosolic CEH was the purification of the enzyme protein. In this study, detergent solubilisation, hydrophobic chromatography and affinity chromatography were investigated in this connection. A procedure for the partial purification of the enzyme from bovine adrenocortical cytosol, using detergent solubilisation was developed and the identification of the enzyme protein was attempted using $[\gamma\text{-}^{32}\text{P}]$ ATP and $[1,3\text{-}^3\text{H}]$ DFP to label the enzyme.

Initial studies with detergents had shown that cholate effectively solubilised the CEH activity in ammonium sulphate precipitates prepared from bovine adrenocortical cytosol. The solubilised enzyme eluted on Sepharose CL-4B chromatography in one peak at $1.8 \times V_0$ indicating particles of uniform and relatively low molecular weight. The cholate solubilised enzyme, so obtained, showed about an 8-fold purification over the tissue cytosol. However, prolonged exposure of the enzyme to cholate at 4°C resulted in significant losses of enzyme activity. This prevented further purification of the enzyme in buffers containing cholate even when the cholate concentration was as low as 0.02%. Furthermore, ion-exchange chromatography was attempted, however cholate, being an ionic detergent, interfered with the protein-adsorbant interactions. It was probable that in the cholate solutions, the enzyme was present in rather homogeneous protein-detergent complexes of similar charge, and that these bind to and then elute en masse from the columns of opposite charge. It is unlikely that cation exchangers would be more successful in the purification of the cholate solubilised enzyme, as similar problems would arise. Although cholate was found to be the most effective detergent in the solubilisation of CEH, other non-ionic detergents, such as nonylglucoside and Nonidet P42 gave encouraging results.

It may be possible to avoid the problems of cholate-induced deactivation and interference in ion-exchange techniques by exchanging the cholate for a non-ionic detergent after the Sepharose CL-4B step. The hydroxyl-apatite step just prior to the ion-exchange steps would be suitable position for such a procedure. However, it is probable that non-ionic detergents will also inhibit the CEH activity (see Berglund, Khoo et al. (1980), Fredrikson, Stalfors et al. (1981a)).

It was thought that hydrophobic chromatography would be a useful technique in the purification of a lipophilic protein such as CEH. However, in this study where a range of ligands were investigated a successful purification was not obtained under the conditions investigated. It was found that the enzyme could be successfully bound to the columns, but it was impossible to elute the bound enzyme using a wide range of elution conditions. It was possible that the enzyme was eluted but in an inactive state. As the identity of the enzyme protein was not known at this time, this could not be investigated. Cholate solubilised enzyme was used in these experiments and so it was possible that cholate was interfering in the protein-adsorbant interactions. Some parallel experiments were performed with cholate solubilised enzyme after dialysis to remove the cholate and this gave identical results (results not shown). However the enzyme activity was unstable after removal of the detergent. These results suggested that hydrophobic chromatography, under the conditions investigated, was of little value in the purification of CEH from bovine adrenocortical cytosol. There have been no other reports on the use of this technique in the purification of adrenal cytosolic CEH or HSL from adipose tissue.

Conventional affinity chromatography techniques are difficult to apply to an enzyme such as CEH, where a hydrophobic substrate is the only biospecific ligand. The problems associated with synthesising a

conventional affinity matrix using cholesterol esters of long chain fatty acids have prevented the use of this technique. However a simple affinity column using glass beads non-covalently coated with cholesterol oleate was developed in this study. CEH was bound to this column at pH 8.4 and could be eluted by reducing the pH and inclusion of cholate in the buffer. The enzyme activity was labile and was unable to be stabilised. No protein could be detected on SDS/PAGE of the concentrated, purified enzyme. One explanation for these results was that the enzyme was in low concentration in the tissue. This, combined with the low capacity of the affinity column, resulted in extremely small amounts of enzyme protein being purified.

In a recent study, where CEH from bovine adrenocortical cytosol was purified 300-fold, in the peak fraction, from a hydroxylapatite column, the CEH represented only 1-2% of the protein in the purified fraction (Cook, Lee and Yeaman (1981)). [^3H] DFP incorporation experiments suggested a 15 000-fold purification would be necessary to purify the enzyme to homogeneity. These results confirmed that CEH was in low concentration in this tissue. Based on these figures the maximum amount of enzyme protein present in the purified fraction from the affinity column would be about 3-4 μg assuming 100% recovery. The results shown in Fig. 4.4 showed that the enzyme was only partly eluted by different conditions, although no detailed study could be performed due to the instability of the enzyme. Therefore it was probable that only 1-2 μg enzyme protein was eluted from the affinity column and this could be lost due to binding to the glass surface and so the activity was quickly lost and no protein was detected on the gels. As it was probable that no protein was loaded onto the gel it is unlikely that the more sensitive silver stain would be of any benefit in these experiments.

The discovery that CEH is present in adrenal cortex in such low concentration has made the need for an effective affinity chromatography

step in the purification of the enzyme even greater. In general, only an affinity technique can give the high purification factors necessary for enzymes in low concentrations. The glass bead column showed that non-covalently coating a suitable surface with cholesterol oleate could be a useful technique, but the columns used here were of low capacity. Attempts to increase the capacity of the column by using larger numbers of smaller beads were met with technical problems, and a stable coating was not achieved. Assuming these problems can be overcome, a large column of small (1 mm) beads with a thin coating of cholesterol oleate may have the capacity to enable the isolation of pure enzyme in an active state. Beads of smaller dimensions may not be suitable for this technique as the thickness of coating may approach the diameter of the bead and this could lead to increased problems with beads associating and affecting the flow rate.

During the course of this study, several reports appeared describing the purification of HSL from chicken adipose tissue (Berglund, Khoo et al. (1980)) and rat adipose tissue (Khoo, Berglund et al. (1980), Fredrikson, Stralfors et al. (1981a)). All these studies included detergent solubilisation and one reported the use of an affinity technique. Fredrikson, Stralfors et al. (1981a) reported a 2000-fold purification of HSL from rat adipose tissue using solubilisation by a non-ionic detergent, $C_{13}E_{12}$, followed by gradient sievorptive chromatography and chromatography on a "triacylglycerol-containing gel". This method was also employed in this study, to determine whether these techniques could be applied to the purification of CEH from adrenal cortex.

The CEH was purified 150-fold from bovine adrenocortical cytosol by a modification of this method up to the second gradient sievorptive chromatography step (CEH_{Q2} preparation). However attempts at affinity chromatography using cholesterol oleate-containing gel or cholesterol

oleate-coated glass beads were unsuccessful in further purification of the enzyme. Labelling experiments with $[\gamma\text{-}^{32}\text{P}]$ ATP and $[1,3\text{-}^3\text{H}]$ DFP indicated that the CEH enzyme protein had Mr 82 000-84 000. This protein was not observed on SDS/PAGE when the gel was stained for protein. This was not surprising as the enzyme protein would only represent 1% of the total protein in this preparation. The silver stain should be able to detect the protein if about 50 μg total protein was loaded on the gel, however it may be masked by the many other proteins in the preparation.

The estimate for the molecular weight of CEH from bovine adrenal was similar to the molecular weight of 84 000 estimated for HSL in rat adipose tissue (Khoo, Berglund *et al.* (1980), Fredrikson, Stralfors *et al.* (1981a)). Furthermore CEH was able to be partially purified from bovine adrenal cortex using similar methods to those used for the purification of HSL from rat adipose tissue. $[1,3\text{-}^3\text{H}]$ DFP labelling of the CEH_{Q2} preparation resulted in two main proteins labelled with Mr 84 000 and approximately 50 000. This was similar to the pattern of labelling found by Fredrikson, Stralfors *et al.* (1981a) when the HSL_{Q2} preparation was labelled with $[1,3\text{-}^3\text{H}]$ DFP. Both enzymes were inhibited to a similar degree by DFP. Incubation of the HSL_{Q2} preparation with $[\gamma\text{-}^{32}\text{P}]$ ATP resulted in only one protein with Mr 84 000 being labelled (Fredrikson, Stalfors *et al.* (1981a)). A similar result was also found in this study with one CEH_{Q2} preparation, although in general several other proteins were also labelled. These results suggested that the CEH activity of adrenal cortex and the HSL activity of adipose tissue were contained on the same enzyme protein.

In another series of studies the HSL from chicken and rat adipose tissue was purified 325- and 300-fold, respectively, using solubilisation with Triton X-100 followed by ion-exchange chromatography (Berglund,

Khoo et al. (1980), Khoo, Berglund et al. (1980)). Although these preparations contained several proteins, $[\gamma\text{-}^{32}\text{P}]$ ATP-labelling studies indicated Mr's of 42 000 and 84 000 for the chicken and rat enzymes respectively. Some preliminary evidence suggested that if the chicken enzyme was treated with SDS buffer for only a short time at room temperature an Mr of approximately 85 000 was obtained on SDS/PAGE (Khoo, Berglund et al. (1980)). Cook, Lee and Yeaman (1981) reported a 60-fold purification of CEH from bovine adrenal cortex using solubilisation by Triton X-100 and ion-exchange chromatography. A major band with Mr 41 000 was observed in this preparation. Incubation of this preparation with $[1,3\text{-}^3\text{H}]$ DFP gave two labelled proteins with Mr 84 000 and 29 000. Further chromatography on hydroxylapatite showed that the enzyme activity cofractionated with the ^3H -labelled protein with Mr 84 000, and was resolved from the Mr 41 000 and 29 000 proteins. Therefore, as before, comparable procedures had been used, with similar results in the purification of HSL from adipose tissue and CEH from adrenal cortex.

Recently, a direct comparative study has been carried out on the HSL from rat adipose tissue, purified by $\text{C}_{13}\text{E}_{12}$ solubilisation and gradient sievorptive chromatography (HSL_{Q2} preparation) and the CEH from bovine adrenal cortex, purified by Triton X-100 solubilisation and ion-exchange chromatography (Cook, Yeaman et al. (1982)). Comparison of substrate specificities, inhibition characteristics, phosphorylation and activation of the two enzymes provided strong evidence that the two activities were catalysed by proteins that were identical to, or very similar to each other.

The CEH_{Q2} preparation contained no phosphoprotein phosphatase activity but did exhibit residual cAMP-dependent and -independent protein kinase activities. A further purification step is required to

remove the protein kinase activity before this preparation could be used in studies of the postulated control mechanism. However the $C_{13}E_{12}$ detergent inhibits CEH, and may have inhibitory effects on phosphoprotein phosphatase and cAMP-dependent protein kinase. Preliminary studies (Section 6) showed that detergent inhibition is likely to prove a problem if the CEH_{Q2} preparation is used as the source of CEH activity for these experiments. It may be possible to exchange the $C_{13}E_{12}$ detergent for a less inhibitory detergent on a hydroxylapatite column, although the two other detergents used with the CEH/HSL enzyme, cholate and Triton X-100, have also been found to deactivate the enzyme on long term exposure (see Section 3.4 this thesis, Berglund, Khoo et al. (1980)). Therefore, although detergent solubilisation has been useful, and indeed, necessary for the purification and identification of the CEH enzyme protein it may prove to be incompatible with detailed enzymological studies of the enzyme.

This thesis contains the first reported study of the effect of a phosphoprotein phosphatase, purified from bovine adrenocortical cytosol, on the activity of bovine adrenocortical cytosolic CEH. An Mr 35 000, multifunctional phosphoprotein phosphatase was purified to a state approaching homogeneity. This enzyme was active when assayed against p-nitrophenyl phosphate, ^{32}P -labelled phosphohistone and ^{32}P -labelled phosphoCEH preparation. Divalent cations were not required for the phosphoprotein phosphatase activity but Mg^{2+} and a thiol such as 2-mercaptoethanol were required for the alkaline phosphatase activity. However, this enzyme did not deactivate CEH in the absence of divalent cations and the addition of low concentrations of Mn^{2+} , Mg^{2+} , Co^{2+} and Ca^{2+} did not affect this result. Higher concentration of Mn^{2+} and Mg^{2+} stimulated endogenous phosphoprotein phosphatases in the CEH preparation used (cholate solubilised enzyme) and made interpretation of results

difficult. Higher concentrations of Ca^{2+} also tended to inhibit the enzyme activity. It was not known if this was a direct effect on the enzyme activity or if it was due to the stimulation of endogenous phosphoprotein phosphatases. There is little known about the effect of Ca^{2+} or calmodulin on the enzyme system.

These results suggested that the low molecular weight phosphoprotein phosphatase or catalytic subunit of the high molecular weight phosphatases was not able to deactivate CEH. It was possible that another subunit, present in the physiological forms of the phosphoprotein phosphatase, was required for the dephosphorylation and deactivation of CEH. High molecular weight forms of phosphoprotein phosphatase have been purified from adrenal cortex, but the activities were not measured against CEH (Kalala, Goris and Merlevede (1973), Ullman and Perlman (1975)). There have been studies into the subunit composition of high molecular weight phosphoprotein phosphatases but little is known about the functions of these subunits (Tamura and Tsuiki (1980), Tamura, Kikuchi et al. (1980)). The role of the phosphoprotein phosphatase with a catalytic subunit, F_c , with Mr 70 000 and an activator protein, F_A , with Mr 50 000 (Yang, Vandenheede et al. (1980), Vandenheede, Yang et al. (1980)) in the regulation of CEH activity in adrenal cortex is unknown and requires further investigation. For these studies a pure preparation of CEH, or one free from endogenous phosphoprotein phosphatase activity, such as the CEH_{Q2} preparation, would be required. This preparation was not available at the time that the experiments described here were performed.

A phosphoprotein phosphatase with Mr 28 000 on Sephadex G-75, was purified from rat adipose tissue via an ethanol precipitation step (Severson and Sloan (1980)). This enzyme was able to deactivate the HSL of rat adipose tissue in a divalent cation-dependent reaction.

No SDS/PAGE evidence of the purity of this enzyme was given, and the relationship of this enzyme to the Mr 35 000 phosphoprotein phosphatase was not known. However, it was possible that the Mr 28 000 phosphoprotein phosphatase preparation contained other protein subunits of the physiological phosphoprotein phosphatase, as it was not purified to the extent that the Mr 35 000 subunit was.

Apolipoprotein A-I was found to inactivate CEH in a dose dependent manner. Prior activation of CEH appeared to increase the sensitivity of the enzyme to the inhibition. There are several possible mechanisms that could explain these results such as: apolipoprotein A-I interacts with the active centre of CEH, and the interaction is enhanced by phosphorylation possibly via conformational changes; apolipoprotein A-I interacts with another site on the enzyme and phosphorylation results in a conformational change which leads to a more exposed allosteric site; apolipoprotein A-I specifically deactivates CEH by dephosphorylation, either directly or via stimulation of a phosphoprotein phosphatase; apolipoprotein A-I interacts with the substrate emulsion to form complexes in which the cholesterol ester is not available to CEH; apolipoprotein A-I sequesters the newly formed fatty acid and prevents its release into the aqueous layer of the assay system.

Studies showed that the apolipoprotein A-I did not contain non-specific phosphoprotein phosphatase activity and did not stimulate phosphoprotein phosphatases. However it was able to interact and disrupt the cholesterol oleate substrate emulsion in the presence of phospholipid. Therefore interaction with the substrate emulsion could partly explain the results. Control tubes with apolipoprotein A-I and cholesterol $[1-^{14}\text{C}]$ oleate substrate emulsion gave similar background readings (which indicate the amount of $[1-^{14}\text{C}]$ oleate in the emulsion) as the substrate emulsion on its own, indicating that apolipoprotein A-I

did not sequester $[1-^{14}\text{C}]$ oleic acid. However this was not tested with $[1-^{14}\text{C}]$ oleic acid standards. It may be possible in the future to further elucidate the mechanism of the apolipoprotein A-I inhibition using purified CEH preparations with ^{125}I -labelled apolipoprotein A-I, $[1,3-^3\text{H}]$ DFP or a radiolabelled substrate analogue and $[\gamma-^{32}\text{P}]$ ATP. The interaction of apolipoprotein A-I with different, labelled forms of the enzyme may determine the nature of the interaction if it is a direct apolipoprotein A-I/CEH enzyme protein interaction.

The physiological significance of such an inhibition is not known. It is possible to speculate that HDL degradation in adrenal cortex could lead to the deactivation of CEH via the production of apolipoprotein A-I. This would prevent depletion of cholesterol ester stores during a prolonged ACTH stimulation when lipoprotein uptake is increased, and allow the build up of cholesterol ester stores following a prolonged ACTH stimulus (see Fig. 1.1). However there is no evidence to suggest that HDL plays a major role in the provision of cholesterol to bovine adrenal glands. Furthermore, although it has been found that apolipoprotein A-I inhibits hepatic triglyceride lipase, the mechanisms and physiological significance of this inhibition are unknown (Kubo, Matsuzawa *et al.* (1981)).

Cytosolic CEH activity in rat adrenal was found to exhibit a diurnal variation in its activity in response to the circadian rhythm of the pituitary-adrenal axis. This is in contrast to an earlier report by Pederson and Brownie (1979) where no difference in CEH activity was found when measured at the high and low points of the circadian cycle. The authors also stated that with sampling at 4 h intervals, a circadian rhythm in CEH activity was either absent or very modest, however no data were shown. Recently, the diurnal rhythm in rat adrenal cytosolic CEH was confirmed by Beins, Vining and Balasubramaniam (1982), who measured the enzyme activity at 10 points in the 24 h period.

In the study here, serum corticosterone was found to reflect the variation in CEH activity. The variation of plasma corticosterone found by Beins, Vining and Balasubramaniam (1982) was similar to the variation observed here. The concentration increased from a minimum in the middle of the light phase to a maximum in the early part of the dark phase, before decreasing in the later part the dark phase back to the minimum. However the variation in adrenal CEH found by Beins, Vining and Balasubramaniam (1982) did not fully correlate with the plasma corticosterone or the known pattern of ACTH secretion. The CEH activity increased from a low point in the middle of the light phase to a high point at the onset of the dark phase, then decreased through the dark phase to another low point before increasing to a further high level at the onset of the light phase. This high level at the onset of the light phase does not reflect the known ACTH secretion (Ixart, Szafarczyk et al. (1977)) and was not reflected in the plasma corticosterone concentration. Recently, a further study has described a diurnal rhythm for cytosolic CEH in rat adrenals when the enzyme activity was measured at 6 points in a 12 h light-12 h dark cycle (Civen, Leeb and Morin (1982)). However the CEH activity was found to be lower during the dark phase than throughout the light phase. This variation does not correlate with the known ACTH and corticosterone rhythms, and is not compatible with the results presented here or by Beins, Vining and Balasubramaniam (1982).

In the study here, the concentration of circulating ACTH was perturbed by prior administration of either corticoid or ACTH in vivo, and the effect on adrenal CEH activity and serum corticosterone concentration was monitored. Acute ACTH administration increased the CEH activity and the serum corticosterone concentration at all points in the light-dark cycle. Furthermore the diurnal variations observed in the control group were almost totally abolished by the ACTH treatment.

These results supported the view that rat adrenal cytosolic CEH activity was under in vivo regulation by circulating ACTH. Furthermore, the CEH activity at all time points was increased to a similar level by the large dose of ACTH, suggesting an upper limit to the enzyme activity.

Dexamethasone treatment to suppress the pituitary secretion of ACTH resulted in significant decreases in both CEH activity and serum corticosterone at all time points. However the pattern of diurnal variation in CEH activity and serum corticosterone was still observed and unchanged. Recently it has been shown that dexamethasone blocked the ACTH response but not the α -MSH response in rat pituitary corticotrophs stimulated by synthetic CRF (Proulx-Ferland, Labrie et al. (1982)). This suggests that only ACTH secretion is suppressed by dexamethasone, and that the secretion of other peptides, derived from the POMC molecule, is not affected. Therefore a reduction in adrenal cytosolic CEH was observed here, probably, in response to a reduction in ACTH alone. Taken together the results indicated that adrenal CEH activity was under in vivo regulation in response to the levels of circulating ACTH, and that the variation in enzyme activity observed was not due to some inherent property of the adrenal cortex.

A simple method was devised for obtaining the lipid droplet fractions from rat adrenal and bovine adrenocortical cytosol. It was found that after two washes the total cholesterol:protein (mg:mg) ratio was about 26:1 indicating that bovine adrenocortical lipid droplets contained about 4% by weight protein. Further washing did not alter this ratio, suggesting that two washes were sufficient to remove loosely associated protein. The similarity or relationship of the lipid droplets, so obtained, to the physiological situation in vivo was unknown. A major apoprotein with a subunit Mr approximately 40 000 was found in both rat and bovine lipid droplets. Several other proteins were observed

including ones with Mr 90 000, 75 000, 65 000, 53 000, 20 000 and 12 000. However, problems were encountered due to the low solubility of the delipidated proteins in aqueous media. This resulted in some inconsistency in the amount and relative proportions of the different apoproteins observed in different experiments. The specific treatments devised for the delipidation of plasma lipoproteins may provide more consistent results, but the method used here led to unacceptable losses of the already small amounts of protein in these preparations. The acetone treatment substantially improved the yields of protein but did not overcome the inconsistency introduced by incomplete resolubilisation of the delipidated protein.

Labelling the protein fractions in whole lipid droplets with $[\gamma\text{-}^{32}\text{P}]$ ATP and cAMP-dependent protein kinase resulted in the phosphorylation of the Mr 40 000 apoprotein in the rat, but not bovine tissue. After delipidation the Mr 40 000 subunit was phosphorylated in both cases. Although, the possible reasons for the lack of labelling in whole bovine lipid droplets were discussed in molecular terms, the physiological significance of this finding was unknown. Several other protein subunits were also phosphorylated, with a similar pattern in rat and bovine tissue, but the physiological role of these phosphorylations and whether they occur in vivo was not known. In preliminary experiments no cAMP-dependent protein kinase activity was detected in the bovine lipid droplets, although phosphoprotein phosphatase activity was observed in these preparations.

Some CEH activity was found to be associated with bovine lipid droplets. The specific activity after two washes was approximately 8 nmol oleic acid produced.min⁻¹.mg protein⁻¹, and subsequent washing did not alter this figure. This suggested that the CEH enzyme protein was a permanent constituent of the protein fraction of bovine adrenocortical

lipid droplets. The specific activity was approximately 10 times that of the enzyme in the cytosol, due to the low protein concentration of the lipid droplets. However, a subunit with Mr 82 000-84 000 was not detected in the lipid droplet protein fractions by $[\gamma\text{-}^{32}\text{P}]$ ATP or $[1,3\text{-}^3\text{H}]$ DFP labelling. This did not constitute a major contradiction as the enzyme in cytosol would require a 15 000-fold purification for homogeneity, and so even in lipid droplets where the enzyme was 10-fold purer the enzyme protein would only constitute 0.075% of the total protein. In most of the labelling experiments this would be about 0.1-0.01 μg CEH enzyme protein. However the amount of protein used in the $[\gamma\text{-}^{32}\text{P}]$ ATP-labelling of whole bovine lipid droplets was higher and possibly some phosphorylation in the Mr 82 000-84 000 region was detected. Preliminary experiments indicated that whole lipid droplets did not significantly alter the activity of CEH in the cytosol, suggesting a protein cofactor for the enzyme activity was not present in the droplets.

10.2 OVERALL SUMMARY

1. Detergent solubilisation was essential for the purification and identification of the CEH enzyme protein. However problems with detergent inhibition in purified preparations may obstruct detailed enzymological studies.
2. The CEH enzyme protein is in low concentration in adrenocortical cytosol. An affinity technique is essential for the further purification of the enzyme.
3. Labelling studies suggested that CEH activity in bovine adrenocortical cytosol was associated with a protein with a subunit of Mr 82 000-84 000. This enzyme protein is probably identical to, or similar to, the HSL enzyme protein found in rat adipose tissue.
4. Apolipoprotein A-I inhibited bovine adrenocortical cytosolic CEH activity, but the mechanism and physiological significance of the inhibition was unclear.

5. A low molecular weight (Mr 35 000) phosphoprotein phosphatase, of the multifunctional phosphoprotein phosphatase type, purified from bovine adrenocortical cytosol, did not deactivate bovine adrenocortical cytosolic CEH.
6. CEH in rat adrenal cytosol exhibits a diurnal variation in its activity in response to the circadian rhythm of the pituitary-adrenal axis.
7. Bovine adrenocortical lipid droplets contain approximately 4% protein by weight. A major apoprotein with a subunit Mr 40 000 was found, which could be phosphorylated in rat but not bovine lipid droplets.
8. CEH was found to be a minor constituent of the lipid droplet protein fraction.

10.3 FUTURE STUDIES

The CEH enzyme protein from bovine adrenocortical cytosol should be further purified using detergent solubilisation and an affinity chromatography technique to confirm the identity of the enzyme protein. New detergents should be investigated to reduce the effects of detergent inhibition on the purified preparations. The in vivo regulation of the enzyme should be further studied in isolated cell systems. Incubation of bovine adrenocortical cell suspensions or cultures with $^{32}\text{P}_i$ under different hormonal and other physiological stimuli could allow the correlation of cytosolic CEH activity with the phosphorylation state of the enzyme in vivo. Due to the low concentration of CEH and the probable large number of phosphorylated proteins, it may be necessary to partially purify the enzyme for these studies.

A physiological, high molecular weight, phosphoprotein phosphatase should be purified from bovine adrenocortical cytosol, and its effect on the dephosphorylation and deactivation of purified cytosolic CEH in vitro, should be studied. The role of the non-catalytic subunits

of the physiological phosphoprotein phosphatase should be investigated. The role of the phosphorylatable phosphoprotein phosphatase inhibitor -1 in the regulation of cytosolic CEH could be studied in vitro and in vivo, in the experiments described above.

The protein fraction of bovine adrenocortical lipid droplets should be further studied to elucidate the role(s), if any, of the apoproteins. The primary task is the development of a reliable delipidation and resolubilisation procedure for the consistent and quantitative recovery of the apoproteins for SDS/PAGE. However it may not be possible to obtain all the apoproteins in aqueous solution, to determine the effect of the apoproteins on the CEH enzyme activity, due to their hydrophobic nature.

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